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GENETIC RECOMBINATIONS LEADING TO PRODUCTION OF ACTIVE BACTERIOPHAGE FROM ULTRAVIOLET INACTIVATED BACTERIOPHAGE PARTICLES[1]

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WITH AN APPENDIX BY R. DULBECCO

Received June 28, 1948

THE potentialities of bacteriophage genetics have been revealed by the discovery of genetic recombinations among related phage particles infecting the same bacterial cell (DELBRÜCK and BAILEY 1946). The complexities of these genetic systems have been further illustrated by the work of HERSHEY and ROTMAN (1948) on a number of different genetic determinants involved in the determination of the alternative phenotypes r^+ and r in phage *T2H*. A different approach to the genetic mechanisms of bacteriophages originated from the chance observation by DELBRÜCK and BAILEY that, after exposure to ultraviolet light, some phages gave variable plaque counts, depending on the relative concentrations of phage lysates and host cells at the time of assay. In investigating this phenomenon, one of us (LURIA 1947) discovered a mechanism of phage reactivation by interaction among inactive particles in the course of intracellular growth. The detailed investigation of this phenomenon has indicated new possibilities for a quantitative analysis of the genetic structure of these viruses and has suggested a possible mechanism for their reproduction.

A preliminary discussion of some of the results reported in this paper has appeared (LURIA 1947); their implications for a number of problems have been discussed in a forthcoming publication (LURIA 1948). The present article is intended to present the results in detail, and, by describing techniques and methods of analysis, to serve as a background for future publications on this topic.

The analysis of the results presented in the following pages is based on the hypothesis that inactivation of bacteriophage particles by ultraviolet light is due to production of discrete alterations in individual portions of genetic material. Although the internal evidence in support of this hypothesis, as presented in this paper, is quite satisfactory, it must be said that satisfactory external evidence from other lines of attack is not yet available. The conclusions reached in this article must be considered for the time being as working hypotheses for further investigation.

MATERIAL AND GENERAL METHODS

The system of phages *T1-T7*, their r mutants, and their common host *Escherichia coli* strain B have repeatedly been described, as well as the use of

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bacterial mutants resistant to one or more phages as indicators for one phage in the presence of another (see DELBRÜCK 1946). Plate counts for viable bacteria, and plaque counts in agar layer for active phage were used throughout, employing 1.1 percent agar in "Difco" nutrient broth plus 0.5 percent NaCl. All plates were incubated at 37°C. Experimental bacterial cultures in the logarithmic phase of growth were grown with aeration at 37°C from standard inocula.

The phage stocks were lysates in glucose+ammonia (or lactate+ammonia) medium. These media give negligible absorption of the ultraviolet light used in this work. High titer phage lysates (over 1×10^{11} particles per ml) might give some ultraviolet screening effect because of bacterial debris and of phage itself. Whenever possible, therefore, phage was irradiated after a dilution 1:5 or higher in the same medium. The source of ultraviolet was a General Electric Company germicidal bulb, 15 watts, alimented through a stabilizer. At a distance of 50 cm from the center of this bulb, the flux—measured with a Westinghouse SM-200 meter with tantalum phototube WL-775—is about 7 erg$\times$mm^{-2} sec^{-1}. The beam contains mainly radiation of wavelength 2537 Å. Samples were irradiated in a thin layer (not over 0.4 mm) in open Petri dishes rocked during exposure.

The technique of "one-step growth" experiment in its various forms has been described in detail previously (DELBRÜCK and LURIA 1942).

EXPERIMENTAL

Inactivation and reactivation of bacteriophages

Plaque counts on phage suspensions exposed to ultraviolet for various lengths of time generally give survival ratios whose logarithms are proportional to the dose, that is, to the time of exposure (see LATARJET and WAHL 1945, and figure 1). The logarithmic rate indicates a one-bit mechanism of inactivation (LEA 1947), and we can assume that the hit consists of the successful absorption of one quantum. The probability that one quantum produces inactivation is, however, very small: for phage *T2*, for example, one inactivating hit is produced by a dose corresponding to almost 10^4 quanta absorbed per particle (M. ZELLE, personal communication). Only one absorption in 10^4 on the average is, therefore, effective, the others probably producing excitations that do not lead to the inactivating effect.

When the average number of effective hits per particle is r, the proportion of active to total phage will be e^{-r}. For $r=1$, $e^{-r}=0.37$; the corresponding dose is the "inactivation dose" in LEA's terminology (1947). If doses are expressed in multiples of the inactivation dose, their values give directly the average number of hits per particle.

Phage particles inactivated by ultraviolet light are adsorbed by bacteria (LURIA and DELBRÜCK 1942). This is detected because adsorption of one particle by a bacterium causes death of the latter. One can, therefore, measure the rate of adsorption of inactive particles from the survival of bacteria in mixtures containing bacteria and irradiated phage in known proportions. If, on the average, x particles are adsorbed per bacterium, a fraction e^{-x} of the

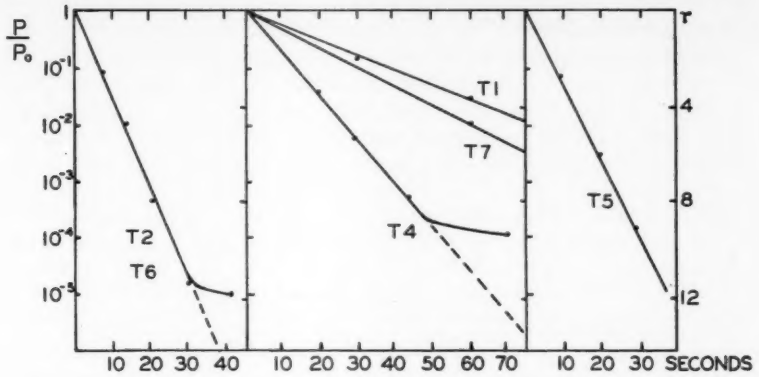

FIGURE 1.—Inactivation of various phages by ultraviolet light. P/P_0=proportion of active phage particles after irradiation. r=ln P_0/P=average number of lethal hits per phage particle. The doses are expressed in seconds of exposure. The deviations for high doses in the curves for *T2*, *T4*, and *T6* are due to reactivation occurring on the assay plates (see text). The broken lines represent extrapolations from the logarithmic portions of the curves.

bacteria should receive no particle and survive. By this means, we could establish that for irradiated phages *T2*, *T4*, *T5*, and *T6*, even for high doses, the rate of adsorption is the same as for unirradiated phage. An experiment of this type is shown in table 1. Only for very high doses a slight reduction occurs in the ability of phage to kill bacteria. This reduction is never such as to require important corrections in the analysis presented in later sections.

With phages *T2*, *T4*, *T5*, and *T6* (the "large particle" phages) the plaque counts on irradiated samples are not independent of the mode of assay. They depend on the concentration of the samples when first mixed with bacteria, in a way illustrated in table 2. In these experiments, bacteria were mixed with various concentrations of irradiated phage. Before lysis and phage liberation

TABLE 1

Killing of bacteria by irradiated phage

0.9 ml of a bacterial culture was mixed with 0.1 ml of each of five suspensions of phage *T2r* that had received various doses of radiation. After 10 minutes, samples were diluted and plated for viable bacterial count.

EXPERIMENT NO.	PHAGE INPUT, PARTICLES PER ML	BACTERIAL INPUT, CELLS PER ML	DOSE OF RADIATION		SURVIVING BACTERIA PER ML	SURVIVAL	PHAGE ADSORBED PER BACTERIUM
			SECONDS	HITS PER PARTICLE		INPUT e^{-x}	x
129	2×10^9	1.15×10^8	0	0	2.3×10^8	0.20	1.60
			60	18	2.4×10^8	0.21	1.56
			70	21	2.2×10^8	0.19	1.66
			80	24	1.6×10^8	0.14	1.96
			100	30	2.7×10^8	0.23	1.47

took place, dilutions were made to bring the total dilution of the irradiated sample to a constant value, and an aliquot plated for plaque count. The plaque counts represent infected bacteria that liberate active phage. Although the total dilution of the irradiated phage on all plates is the same, it is seen that the plaque counts are higher when bacteria have first been placed in contact with a more concentrated phage lysate.

This means that bacteria may produce active phage if they pick up the ir-

TABLE 2

Dependence of plaque counts on irradiated phage T6r on the concentration of the phage sample that is mixed with bacteria

A sample of phage *T6r* containing 1.5×10^{10} particles/ml was irradiated for 20 seconds. The bacterial suspension (B) contained 2×10^{9} cells/ml. Each plate received 0.05 ml of phage dilution and 0.2 ml of suspension (B).

EXPERIMENT NO.	MIXTURE NO.	PROCEDURE	DILUTION OF PHAGE WHEN FIRST MIXED WITH (B)	TOTAL DILUTION FROM THE ORIGINAL PHAGE TO THE SUSPENSION FROM WHICH SAMPLES ARE PLATED	PLAQUE COUNT (SUM OF TWO PLATES)
5	1	0.1 ml *T6r*→0.9 ml (B); kept 10 min. at 37°C; diluted $1:10^3$, 0.05 cc plated	1:10	$1:10^4$	1318
	2	0.1 ml (*T6r* 1:10)→0.9 ml (B); kept 10 min. at 37°C; diluted $1:10^2$, 0.05 ml plated	$1:10^2$	$1:10^4$	474
	3	0.1 ml (*T6r* $1:10^2$)→0.9 ml (B); kept 10 min. at 37°C; diluted 1:10, 0.05 ml plated	$1:10^4$	$1:10^4$	250
	4	0.05 ml (*T6r* $1:10^4$) plated	Less than $1:10^5$ (on plate)	$1:10^4$	57

radiated particles from a concentrated phage suspension, but not from a dilute one. The immediate explanation is that from a concentrated lysate the bacteria receive some other "factor," which, inside the bacterium, somehow *reactivates* an "inactive" particle and which is not present in dilute lysates. An "inactive" particle can be defined as one that has lost the ability to initiate production of active phage unless adsorbed by a bacterium together with the unknown "factor."

Reactivation still occurs after storage of irradiated phage for weeks in an ice-box. Reactivation gives rise to fully active phage particles. This can be proved either by sampling phage from the plaques or by letting the bacteria, in which reactivation occurs, lyse in liquid and then testing the lysate for active particles.

The occurrence of reactivation for certain phages accounts for deviations from the logarithmic inactivation rate found for these same phages (see figure 1). As the dose of radiation increases beyond a certain point, the survival, as determined by plaque count, appears to diminish less rapidly. This is due to an unavoidable partial reactivation. In a phage titration, we mix approximately 5×10^7 bacteria with an amount of phage suspension such as to give approximately 100 plaques, and pour the mixture on an agar plate. On the one hand, if we are dealing with a fully active sample, the bacteria only come in contact with 100 active particles. On the other hand, in assaying an irradiated suspension containing, for example, one active particle in 10^6, we expose the bacteria to 10^8 inactive particles plus a correspondingly large amount of other lysate constituents, besides the residual 100 active particles. Conditions permitting reactivation, therefore, obtain in these plating mixtures, and reactivation disturbs and often completely obscures the count of the residual active phage. For this reason, the survival of fully active phage for high doses must be obtained by extrapolation from the logarithmic part of the curve, a necessarily inefficient procedure. If deviations in the survival rate for high doses occurred, this extrapolation would not be justified. One possible cause of error—screening of some phage particles from radiation by components of the lysate itself—was excluded by irradiating concentrated phage *T6* mixed with phage *T1*, and testing for the inactivation rate of the latter, which is not disturbed by reactivation phenomena. The inactivation rate of *T1* remains the same as in the absence of *T6* up to doses that correspond to 100 hits per particle of phage *T6*.

Identification of the reactivating factor

What is the "factor" present in irradiated stocks of phages *T2*, *T4*, *T5*, or *T6*, which, if acting on bacteria that have adsorbed inactive phage particles, allows production of active phage? Since phage stocks are lysates produced by lysis of the common host *E. coli* B, the factor might be either of phage or of bacterial origin.

The factor was identified as being phage itself, inactive or active, in that *reactivation occurs in bacteria that adsorb either more than one inactive particle of a given phage, or one inactive particle of one phage plus some active or inactive particles of a related phage.* The evidence for this conclusion, which is illustrated in part by the data in table 3, can be summarized as follows.

(a) Addition of an excess of supernatant from a heavy bacterial culture to a mixture of dilute irradiated phage plus bacteria gives no increase in plaque count. The factor in the lysates is not a normal bacterial secretion.

(b) Concentrated lysates of phages *T1*, *T5*, or *T7* added to a mixture of bacteria and dilute irradiated *T2* (or *T4*, or *T6*) do not cause reactivation. No heterologous lysate causes reactivation of *T5*. The test for cross-reactivation is done by plating the mixtures, before lysis, with bacterial indicator strains sensitive to the phage whose reactivation is tested, but not to the others. Since all phage stocks are lysates of common host cells, the factor is not an unspecific bacterial product liberated upon lysis.

TABLE 3

Reactivation of phage T2 under various conditions

Phage *T2*, containing 2×10^{10} particles per ml, was irradiated for 35 seconds. Phages *T6* and *T4*, containing 4×10^{10} particles per ml, were irradiated for 30 seconds. Phages *T1* and *T5* contained 3×10^{10} particles per ml. The bacterial suspensions (B) and (B/6) contained 10^9 cells per ml.

MIXTURE NO.	CONTENTS	AFTER 10 MINUTES	PLAQUE COUNT (SUM OF TWO PLATES)	REACTIVATION
1	0.1 ml Phage *T2* dil. 1:500+1.9 ml (B)	0.05 ml plated with B	22	−
2	0.1 ml Phage *T2* dil. 1:500+1.9 ml (B/6)	0.05 ml. plated with B/6	20	−
3	0.1 ml Phage *T2* pure +1.9 ml (B)	dil. 1:500, 0.05 ml plated with B	4000	+
4	0.1 ml Phage *T2* pure +1.9 ml (B/6)	dil. 1:500, 0.05 ml plated with B/6	3000	+
5	0.1 ml Phage *T2*. dil 1:500+0,1 ml irrad. *T6*+1.8 ml (B)	0.05 ml plated with B/6	670	+
6	0.1 ml Phage *T2* dil. 1:500+0.1 ml unirrad. *T6*+1.8 ml (B)	0.05 ml plated with B/6	190	+
7	0.1 ml Phage *T2* dil. 1:500+0.1 ml irrad. *T4*+1.8 ml (B)	0.05 ml plated with B/4	661	+
8	0.1 ml Phage *T2* dil. 1:500+0.1 ml irrad. *T6*+1.8 ml (B/6)	0.05 ml plated with B/6	10	−
9	0.1 ml Phage *T2* dil. 1:500+0.1 ml unirrad. *T6*+1.8 ml (B/6)	0.05 ml plated with B/6	12	−
10	0.1 ml Phage *T2* dil. 1:500+0.1 ml irrad. *T4*+1.8 ml (B/6)	0.05 ml plated with B/4	883	+
11	0.1 ml Phage *T2* dil. 1:500+0.1 ml unirrad. *T1*+1.8 ml (B)	0.05 ml plated with B/1, 5	18	−
12	0.1 ml Phage *T2* dil. 1:500+0.1 ml unirrad. *T5*+1.8 ml (B)	0.05 ml plated with B/1, 5	17	−

(c) Concentrated lysates of phages *T2* (or *T4*, or *T6*), whether fully active or irradiated, can reactivate irradiated particles of any other *T-even* phage. These phages are morphologically, serologically, and probably genetically related, whereas *T5* belongs to a fully separate group (DELBRÜCK 1946). The "factor" in the lysates appears to carry the same pattern of relatedness. It is not produced by irradiation, since its presence can be proved in unirradiated lysates by the technique of cross-reactivation.

(d) Reactivation is independent of contact between phages prior to infection of the host: a mixture of bacteria and phages gives the same amount of reactivation independently of how long the phages have been together before adding the bacteria.

(e) Phage *T4r* purified by fast centrifugation (kindly supplied by DR. T. F. ANDERSON) shows both self-reactivation as a function of concentration in the mixtures, and ability to reactivate phages *T2* or *T6*. The factor, therefore, is present in such a purified phage suspension.

(f) Reactivation of phage *T2* by lysates of *T6*, for example, only occurs in presence of bacteria capable of adsorbing both phages. Inactive phage *T2* in presence of bacteria *B/6*, by which it is adsorbed, is not reactivated by *T6*, which is not adsorbed, but is reactivated by *T4*, which is adsorbed. This proves

that the "factor" has the same host specificity as the phage in whose lysate it is found.

The last point, particularly, was considered crucial in showing that production of active phage from an inactive particle was actually due to infection of the same bacterial cell with other particles, either of the same or of a different but genetically related phage, active or inactive. Further confirmation came from the experiments discussed below, which showed that in a mixture of inactive phage and bacteria the number of bacteria yielding active phage is never greater than the number of bacteria that adsorb two or more inactive particles, and in some cases actually equals it. The same holds true for reactivation of an inactive phage, for instance *T2*, by a related one, for instance *T4*; the number of bacteria liberating active *T2* is lower than—or equal to—the number of bacteria receiving at least one particle of each phage. The analysis of cross-reactivation between these different wild-type phages will not be discussed further in this paper, but will form the subject of a future publication.[2]

(g) All bacteria which, after infection with inactive particles, do not liberate active phage also fail to lyse. This was proved by mixing bacteria and inactive phage under conditions in which some reactivation occurs, plating a sample of the mixture for plaque count, and another sample for direct microscopic observation of lysis on agar. The results of such experiments proved that the fraction of bacteria that are lysed is the same as the fraction of bacteria that liberate active phage. The other infected bacteria fail to grow and divide, and can be seen still apparently unchanged 24 hours later.

Reactivation and genetic transfer

The limitation of cross-reactivation to the *T-even* phages immediately brought out a similarity between this phenomenon and that of genetic transfer described by DELBRÜCK and BAILEY (1946). In the latter case, bacteria simultaneously infected with the phages $T2r^+$ and *T4r*—the *r* character being the result of mutation from the wild-type, which can be designated as r^+—liberate a mixture of particles of the four types, $T2r^+$, *T2r*, $T4r^+$, and *T4r*, among which the second and third represent new types. These must owe their origin to some sort of recombination involving the genetic determinants for the alternative r^+ and *r* phenotypes. Evidence for the discrete nature of these determinants has since been reported by HERSHEY and ROTMAN (1948).

We assumed then, as a working hypothesis for the analysis of the reactivation phenomenon, that inactivation by ultraviolet light resulted from "lethal mutations" in a number of discrete genetic determinants among inactive particles in the same bacterium to reconstitute fully active particles. This hypothesis can be formulated quantitatively in terms of measurable

[2] Cross-reactivation between *T*-even phages has the limitation that the individual phages are distinguishable only by test of differential properties such as ability to grow on different hosts and rate of inactivation by different antisera. Cross-reactivation can only be defined as the production, upon mixed infection, of active particles having the distinctive properties of an inactive parent particle.

quantities by making a number of simple assumptions. We shall first develop this simple theory and then describe the experiments by means of which it was tested.

Theory

We shall assume that in each particle of a given phage there exist n "units" (or "loci") each capable of undergoing a lethal mutation when exposed to ultraviolet light. Since one effective hit is sufficient to inactivate a phage particle, a lethal mutation can be defined as an effective hit, that is, as an alteration of one unit which makes the phage particle unable to initiate by itself the production of active phage in a bacterium. A particle may undergo more than one lethal mutation, and mutations will be distributed at random and independently among the different units, the distribution depending only on the sensitivity of each unit.

We shall now make the assumption that the sensitivity of all units is the same, and show later that this assumption, if incorrect, only requires a numerical correction which does not invalidate the applicability of the theory.

Our next assumption is that *active phage cannot be produced in a bacterium unless the infecting particle or particles, taken as a group, contain at least one copy of each unit in non-lethal form.* This assumption is an essential feature of the theory, and corresponds to treating each unit as a *discrete, material, independent hereditary unit* endowed with genetic continuity and individuality. An inactive unit cannot be replaced by copies of different units. This assumption implies that production of active units cannot result from the cooperation of two or more lethal units, but only from actual reproduction of active units.

When a population consisting of M phage particles is irradiated with a given dose, there will be produced in each particle, on the average, r lethal mutations, or a total of $M \times r$ mutations in the whole population. Since M particles contain $M \times n$ units, each unit will receive on the average $Mr/Mn = r/n$ lethal mutations.

A given unit, taken at random, will have a probability $e^{-r/n}$ of not having a lethal mutation, and a probability $(1-e^{-r/n})$ of having at least one.

We ask next: what is the probability that each of the n units is present in at least one non-lethal copy in a group of k particles that enter a bacterium? According to our assumptions, this probability should represent an upper limit for the probability that a bacterium produces active phage.

If a bacterium is infected by k particles, the probability that a given unit is lethal in all of them is: $(1-e^{-r/n})^k$, and the probability that it is non-lethal in at least one of them is: $1-(1-e^{-r/n})^k$.

The probability that at least one non-lethal copy of each of the n units is present in the k particles is the product of the probabilities referred to the individual units. Since we have assumed equal sensitivity for all units—that is, r/n constant for all units for each value of r—the product will be

$$[1 - (1 - e^{-r/n})^k]^n. \qquad (1)$$

The expression (1) represents the probability that a bacterium infected by k particles receives at least one full non-lethal complement of the n units.

In a mixture of phage with bacteria, however, there is a distribution of the number of phage particles infecting individual bacteria. With relatively good approximation—see Appendix—this distribution can be considered as a Poisson distribution. If x is the average number of particles adsorbed per bacterium, the fraction of bacteria with k particles is: $x^k e^{-x}/k!$

The fraction of bacteria receiving k particles which carry a full complement of non lethal units is then:

$$\frac{x^k e^{-x}}{k!} [1 - (1 - e^{-r/n})^k]^n. \tag{2}$$

Finally, the fraction of the total bacterial population which receives all units in a non-lethal form is the sum of the expression (2) for all possible values of k:

$$Z = \sum_{k=0}^{\infty} \frac{x^k e^{-x}}{k!} [1 - (1 - e^{-r/n})^k]^n. \tag{3}$$

This expression embodies the following consequences of our hypothesis:

(a) No full complement of active units can be present in uninfected bacteria ($k=0$).

(b) Of the bacteria with one phage particle ($k=1$), only those with an active particle fulfill the requirement for active phage production ($Z=xe^{-x}e^{-r}$).

(c) Any bacterium that receives at least one active particle fulfills the requirement for active phage production, whether it also receives inactive particles or not. For each unit of that particle, $1-e^{-r/n}=0$; hence, $(1-e^{-r/n})^k=0$, and $[1-(1-e^{-r/n})^k]^n=1$.

(d) For any given value of $r>0$, Z increases with increasing x, that is, the probability of having a full complement of active units increases as the number of particles adsorbed per bacterium increases.

(e) For any given value of x, Z diminishes with increasing r, that is, the probability of having all active units diminishes as the dose of radiation increases.

For the purpose of comparison with data from different experiments, it is more convenient to eliminate from the computation those bacteria that receive either zero or one phage particle, since they are not expected to contribute to reactivation. This is done by using instead of Z the expression

$$z = \sum_{k=2}^{\infty} \frac{x^k e^{-x}}{k!} [1 - (1 - e^{-r/n})^k]^n. \tag{4}$$

z represents the fraction of bacteria in the total population that have two or more phage particles, which together contain a full complement of active units. Since the fraction m of bacteria with two or more phage particles ("multiple-infected bacteria") is

$$m = 1 - (x + 1)e^{-x} \tag{5}$$

the multiple-infected bacteria receiving a full complement of active units represent a fraction

$$y = z/m = \frac{\sum_{k=2}^{\infty} \frac{x^k e^{-x}}{k!} [1 - (1 - e^{-r/n})^k]^n}{1 - (x + 1)e^{-x}}. \qquad (6)$$

The expression y thus obtained is a function of x (average number of phage particles adsorbed per bacterium), of r (average number of lethal hits per particle), and of n (number of units per particle).

We shall call w the ratio between the number of bacteria that actually liberate active phage (plaque count) and the number of multiple-infected bacteria. For each mixture of bacteria and irradiated phage, we can determine experimentally r, x, and the plaque count, and obtain from these the values of m and w. We can then compare the experimental values of w with the calculated values of y for several different values of n.[3]

The function $y = F(r, x, n)$ was tabulated numerically for a range of values of r (between 3 and 50), of x (between 0.05 and 20), and of n (between 10 and 60). We used for k those ranges of values for which the contributions of the corresponding classes were relevant. The corresponding curves were drawn for $y = F(r)$ (x and n constant), and for $y = F(x)$ (r and n constant).

Before comparing the experimental results with the curves, it is useful to discuss briefly what we may expect from the comparison. If reactivation only occurs in bacteria with more than one inactive particle, w should never be greater than unity. If among the requirements for reactivation there are those stated in the assumptions of our theory, the ratio w/y should never be greater than unity. Finally, if the requirements stated in our assumptions are *necessary* and *sufficient* for reactivation, the ratio w/y should be unity, that is, active phage should be produced in all those bacteria that receive a full complement of the hypothetical units in non-lethal form. Should this obtain, it would then be possible to calculate the value of n for each phage from the experimental values of w.

It is important to keep in mind that the assumptions of our theory, up to this point, do not contain any implication as to the nature, properties, or mechanism of transfer of the postulated units. They only assert that each unit has genetic individuality and can be made lethal by radiation as a result of one photochemical reaction, which is of the "all or none" type and independent of other reactions of the same type in other units of either the same or other phage particles. Production of active phage is conditioned by the presence in one bacterium of one active copy of each unit, this copy not being replaceable by any number of inactive copies.

The theory does not imply that all phage particles receive the same number of lethal hits, but that the lethal hits are distributed at random among the units of all phage particles.

[3] In preliminary reports (LURIA 1947, 1948) we used the symbol y both for the theoretical and experimental probabilities of reactivation. We also gave values of $1/y$ instead of y(or w). The present notation, while consistent with the previous one, makes the presentation more logical.

Comparison of theory with experiment

Quantitative experiments consisted of testing mixtures of bacteria and irradiated bacteriophage for the number of bacteria that liberate active phage. Only phages *T2*, *T4*, and *T6* were studied in detail.

In a typical experiment, such as the one described in detail in table 4, a standard culture of bacteria, grown to a titer of either 10^8 cells per ml or 10^9 cells per ml, was chilled by immersion in a water bath at 5–6°C. This treatment interrupts multiplication without changing the ability to resume immediate multiplication and to support normal growth of phage upon return to 37°C. In experiments with bacteria grown to a titer of 10^9 (latest part of logarithmic growth phase), immersion in ice-water is not necessary, since multiplication stops almost immediately upon interruption of aeration and transfer to room temperature.

Ten minutes after interrupting multiplication, a sample of the culture is diluted and assayed for viable count. If several mixtures of bacteria and phage are to be prepared, the culture may have to be used for one or two hours, in which case at least one other similar assay is made at the end of the experiment to make sure that no proliferation has occurred. At intervals, undiluted samples of the culture are placed into test tubes, and a constant volume of irradiated (or control) phage variously diluted is added. In this way, we know for each mixture the input of bacteria and of phage per ml. For irradiated samples, the input of active phage is determined from one or more assays done at very high dilution. When this is impossible—for high doses of radiation—the amount of active phage is determined by extrapolation from the first part of the inactivation curve.

Adsorption is interrupted by heavy dilution after a short time (generally five or ten minutes). The amount of adsorption is determined either by determination of free phage in the supernatant of a centrifuged sample from a mixture containing active phage, assuming similar adsorption in all other mixtures (see table 1), or by determining the bacterial survival in samples from various mixtures. With the *T-even* phages, 80 to 95 percent of the phage is adsorbed in ten minutes. The value for the multiplicity of infection, x, for each mixture is used in calculating the fraction m of bacteria with two or more phage particles: $m = 1 - (x+1)e^{-x}$.

Before lysis begins, a suitably diluted sample of the mixture is plated with an excess of sensitive bacteria for plaque count. The plaque count—corrected, when necessary, for the active phage by subtracting the value corresponding to the latter—is divided by m to obtain the experimental value w for that mixture (see table 4).

A large number of experiments, yielding a total of over 1000 values of w, for various doses of radiation and for different multiplicities, were done with the *T-even* phages. Phage *T5* was only partially investigated, because of difficulties in obtaining reproducible results in view of the low and irregular adsorption rate for this phage.

The data for phages *T-even* include those for several of their *r* mutants,

TABLE 4

Reactivation of phage T2r in a series of mixtures of irradiated phage with bacteria. Sample experiment, being a part of Experiment No. 155

Culture of *E. coli B* chilled at 6°C = (B). Bacterial titer by plate count: 1.1×10^8 cells per ml.
Phage *T2r* (stock No. 18c diluted 1:4) irradiated for 20, 30, 40, and 50 seconds.
Control phage, diluted $1:10^7$; 0.1 ml gives 220 plaques. Titer = 2.2×10^{10}
Phage irradiated 20 sec., dil. $1:10^4$; 0.1 ml gives 483 plaques. Titer = 4.8×10^7. Survival (e^{-r}) = 2.2×10^{-3}. r = 6.1.
Phage irradiated 30 sec. dil. $1:4\times10^3$; 0.1 ml gives 36 plaques. Titer = 1.45×10^8. Survival (e^{-r}) = 6.6×10^{-5}. r = 9.6.

MIXTURE NO.	CONTENTS	PLAQUES PER ML OF MIXTURE (SAMPLES PLATED AFTER 10 MINUTES AT 37°C, WITH SUITABLE DILUTION)	PHAGE INPUT	ADSORBED PHAGE*	MULTIPLICITY OF INFECTION x	MULTIPLE-INFECTED BACTERIA m	PLAQUE COUNT**	PLAQUE COUNT / m = w
1	0.1 ml (Phage irrad. 20 sec. dil. 1:2.5)+0.9 ml (B)	9×10^7	8.8×10^8	8.2×10^8	8.2	1×10^8	9×10^7	0.9
2	0.1 ml (Phage irrad. 20 sec. dil. 1:10)+0.9 ml (B)	2×10^7	2.2×10^8	2.05×10^8	2.05	5.5×10^7	2×10^7	0.36
3	0.1 ml (Phage irrad. 30 sec. dil. 1:2.5)+0.9 ml (B)	7.5×10^7	8.8×10^8	8.2×10^8	8.2	1×10^8	7.5×10^7	0.75
4	0.1 ml (Phage irrad. 30 sec. dil. 1:10)+0.9 ml (B)	1×10^7	2.2×10^8	2.05×10^8	2.05	5.5×10^7	1×10^7	0.18
5	0.1 ml (Phage irrad. 30 sec. dil. 1:100)+0.9 ml (B)	2.2×10^5	2.2×10^7	2.05×10^7	0.20	1.6×10^6	2.2×10^5	0.14
6	0.1 ml (Phage irrad. 40 sec. pure)+0.9 ml (B)	8.8×10^7	2.2×10^9	2.05×10^9	20.5	1×10^8	8.8×10^7	0.88
7	0.1 ml (Phage irrad. 40 sec. dil. 1:2.5)+0.9 ml (B)	4.3×10^7	8.8×10^8	8.2×10^8	8.2	1×10^8	4.3×10^7	0.43
8	0.1 ml (Phage irrad. 40 sec. dil. 1:10)+0.9 ml (B)	4.7×10^6	2.2×10^8	2.05×10^8	2.05	5.5×10^7	4.7×10^6	0.085
9	0.1 ml (Phage irrad. 40 sec. dil. 1:100)+0.9 ml (B)	4×10^4	2.2×10^7	2.05×10^7	0.20	1.6×10^6	4×10^4	0.025
10	0.1 ml (Phage irrad. 50 sec. pure)+0.9 ml (B)	5.4×10^7	2.2×10^9	2.05×10^9	20.5	1×10^8	5.4×10^7	0.54
11	0.1 ml (Phage irrad. 50 sec. dil. 1:2.5)+0.9 ml (B)	2.3×10^7	8.8×10^8	8.2×10^8	8.2	1×10^8	2.3×10^7	0.23
12	0.1 ml (Phage irrad. 50 sec. dil. 1:100)+0.9 ml (B)	5.4×10^3	2.2×10^7	2.05×10^7	0.20	1.6×10^6	5.4×10^3	0.0034
13	0.1 ml (Phage Control dil. 1:100)+0.9 ml (B)	2.1×10^7						
	Free in supernatant	1.5×10^6						

* *Determination of adsorption.* Mixture No. 13:
Adsorbed phage = $2.2\times10^7-1.5\times10^6=2.05\times10^7$
Fraction adsorbed = 0.93
Bacteria per tube = 1×10^8
Multiplicity of infection = 0.205
Infected bacteria (calculated) = 1.85×10^7
Infected bacteria + free phage = 2.0×10^7
Plaque count (experimental) = 2.1×10^7

** No correction is made for active phage in the input, since this correction would only affect the second (or higher) decimal figure.

TABLE 5

Probability of reactivation for various phages as a function of dose and of multiplicity of infection

Values of the ratio w between bacteria that yield active phage and multiple-infected bacteria.

EXPERIMENT NO.	PHAGE	MULTIPLICITY x	AVERAGE NUMBER OF HITS PER PARTICLE r						
			2.6	4.8	6.0	9.5	10.0	12.5	15.8
		0.2				0.14		-0.025	0.0034
		0.4						0.028	0.0055
		1						0.055	0.01
		2			0.36	0.18		0.085	
155	*T2r*	4						0.18	0.08
		8			1.0	0.83		0.48	0.26
		20						0.98	0.6
		0.1		0.56	0.42		0.1		
		0.2	(1.0)	(0.83)	0.40		0.1		
		1	(0.9)	(0.59)	0.44		0.17		
		2			0.5		0.23		
156	*T2r*	4			0.67		0.43		
		8			1.05		0.74		
		20			1.0		1.0		
			r 3.6	6.1	7.9	9.6	11.7	12.8	15.5
		0.2				0.065			
		0.4		0.15		0.075		0.03	
		0.8		0.21		0.1		0.035	
162	*T6r*	2		0.27		0.18		0.05	
		4		0.59		0.26		0.18	
		8		0.83		0.67		0.37	
		0.35	(1)		0.27		0.07		0.016
		0.7	(1)		0.37		0.08		0.016
70	*T6*	1.4			0.37		0.12		0.04
		2.8			0.37		0.14		0.06
		5.6			0.48		0.21		0.08
			r	5			10		
		0.35		0.4			0.027		
160	*T4*	0.7		0.29			0.042		
		1.4		0.28			0.06		
		2.8		0.42			0.13		
		7		0.8			0.38		
		14		1.0			0.77		

The values in parentheses are from separate experiments.

No correction is made for active phage in the input, since this correction would only affect the [illegible]

which were found to have the same probability of reactivation as the respective wild types. The results cover ranges of values of r from 2.5 to over 30, and of x from 0.02 to 20.

The individual values of w, and those of the variables, r and x, are obtained from the following actual measurements: 1) titer of phage; 2) total number of bacteria; 3) survival of phage; 4) survival of bacteria and/or assay of free phage; 5) plaque count from the mixture. Each of these measurements involves an error of estimation due to dilution and sampling errors. Several of these determinations, however, are the same within each experiment. The results from individual experiments are, therefore, more consistent than those from different experiments, as shown in table 5.

The only graphic representation that could show all values of w for each phage and allow of comparison with the calculated values of y would be a tri-dimensional plot of w as a function of r and of x. As second best choice, we plotted the values of w as a function of r for several values of x taken as constant, and as a function of x for several values of r taken as constant. Individual values of w fluctuate rather widely, but the data as a whole make it possible to draw curves, which represent averages and which can be considered as the curves for w as a function of r and of x. A number of such plots using all the experimental points for the corresponding values of the variables, are presented in figures 2, 3, and 4 (multiplicity of infection as variable) and figures 5, 6, and 7 (dose of radiation as variable).

The trend of these plots is similar to that of the theoretical curves for y, and it is possible to find for each phage a constant value of n (number of units) such that the corresponding values of y become very similar to those of w for low values of x and for any value of r. That is, it is possible for each phage to determine a constant number of units for which the experimental probability of reactivation equals the theoretical one for any dose of radiation provided the multiplicity of infection is low. The corresponding theoretical curves for y have been drawn in the plots of the values of w. For phage *T2*, the best fit is for $n=25$; for *T4*, $n=15$; for *T6*, $n=30$ (see especially figures 5, 6, and 7).

The main feature emerging from the curves in figures 2, 3, and 4 is that the values of w tend to unity for increasing multiplicity of infection. In several cases, the number of cells that liberate phage actually reaches the number of multiple-infected cells, but in no case does it go beyond it, proving that reactivation does not occur in single-infected cells.

The curves in figures 5, 6, 7, for w as a function of r, are of the multiple-hit type, indicating that suppression of phage production depends on damage in a number of elements. The values of w tend to unity for low doses, again showing that reactivation potentially can take place in every multiple-infected cell.

Comparison in figures 2–4 with the curves for y, chosen to fit the experimental curves for low values of x, shows that the general similarity is limited by a systematic deviation. As the multiplicity increases, both w and y tend asymptotically to unity, but w increases more slowly. This means that, as the number of phage particles per bacterium increases, the probability of reacti-

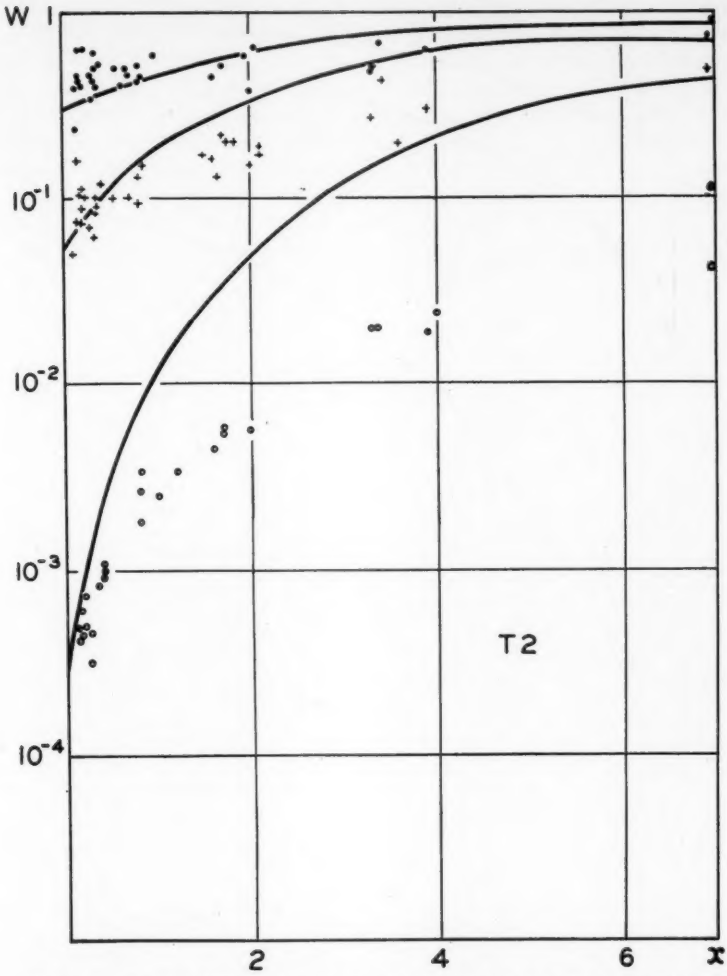

FIGURE 2.—The probability w of reactivation for irradiated phages *T2* and *T2r* as a function of the multiplicity of infection x, for several doses of radiation. Abscissae: values of x. Ordinates: values of w.

● values of w for $r = 6$ hits per particle.
\+ values of w for $r = 10$ hits per particle.
○ values of w for $r = 20$ hits per particle.

Solid lines: theoretical curves for y as a function of x for $n = 25$, and for the values of r given above.

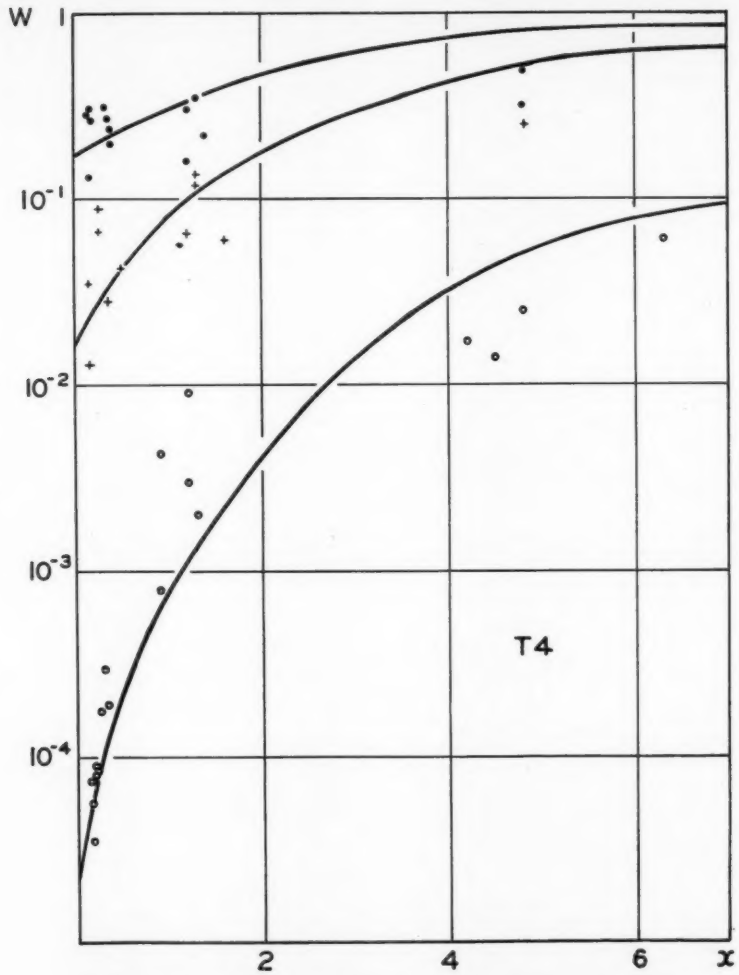

FIGURE 3.—Same as figure 2, for phages *T4* and *T4r*. The theoretical curves for y correspond to $n=15$.

vation, while steadily increasing, does not keep pace with the theoretical function y. The cooperation within groups consisting of more than two particles is not as successful in bringing about reactivation as required by the simple theory, whereas pairs of particles apparently collaborate with an efficiency of one hundred percent.

The deviation for higher multiplicities is reflected in the curves of figures 5–7. The curves for w as a function of the dose are very close to the theoretical

curves for low multiplicities, up to $x=0.5$; for higher multiplicities they fall below the corresponding curves for y calculated for the same number of units. We can actually find, for each value of x, a curve for y—for the same n but corresponding to a lower x—which fits the experimental curve. The corresponding theoretical curves are drawn in figures 5–7. This indicates that groups of more than two particles collaborate in reactivation as if they consisted of a lower but definite number of particles.

It is interesting to notice that the systematic deviation from theory for

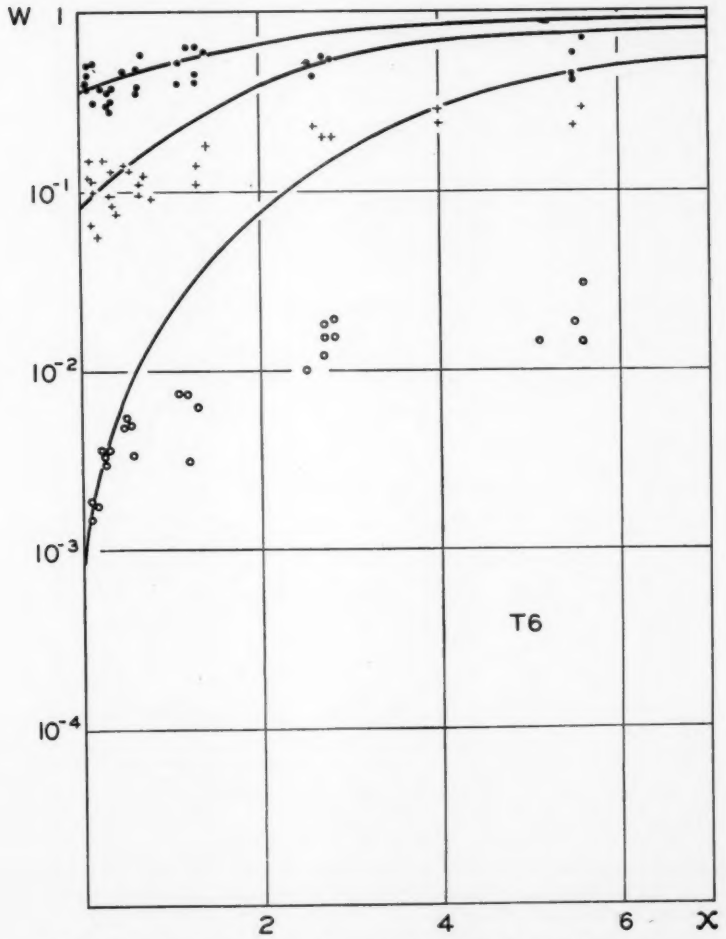

FIGURE 4.—Same as figure 2, for phages *T6* and *T6r*. The theoretical curves for y correspond to $n=30$.

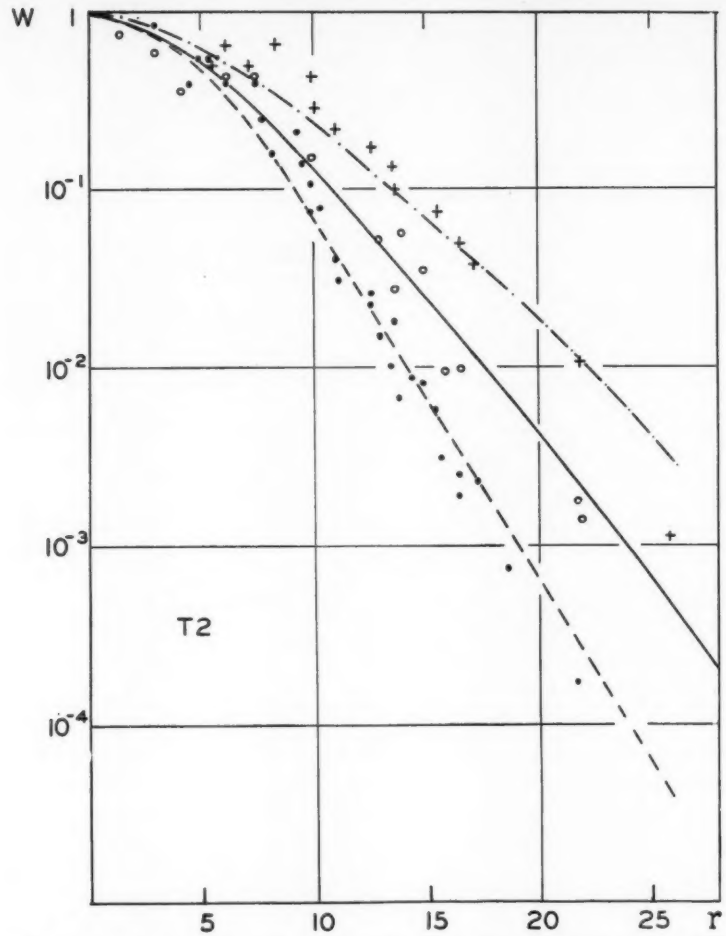

FIGURE 5.—The probability w of reactivation for phages *T2* and *T2r* as a function of the dose of irradiation r (in hits per particle) for several multiplicities. Abscissae: values of r. Ordinates: values of w.

● values of w for x=0.1–0.2.
○ values of w for x=0.8–1.5.
\+ values of w for x=2.5–4.0.

Broken line: theoretical curve for y as a function of r for n=25, x=0.15. Solid line: theoretical curve for y as a function of r for n=25, x=0.6. Broken and dotted line: theoretical curve for y as a function of r for n=25, x=1.3.

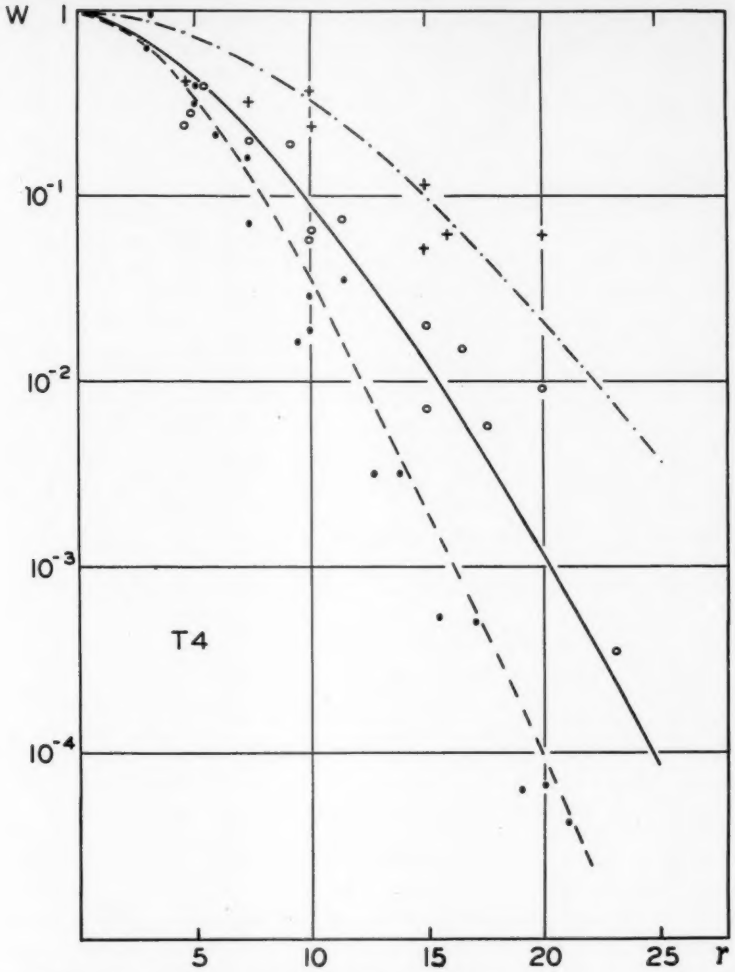

FIGURE 6.—Same as figure 5, for phages *T4* and *T4r*.

● values of w for x=0.1–0.2.
○ values of w for x=0.8–1.5.
\+ values of w for x=4–7.

Broken line: curve of y for n=15, x=0.15. Solid line: curve of y for n=15, x=1.1. Broken and dotted line: curve of y for n=15, x=4.0.

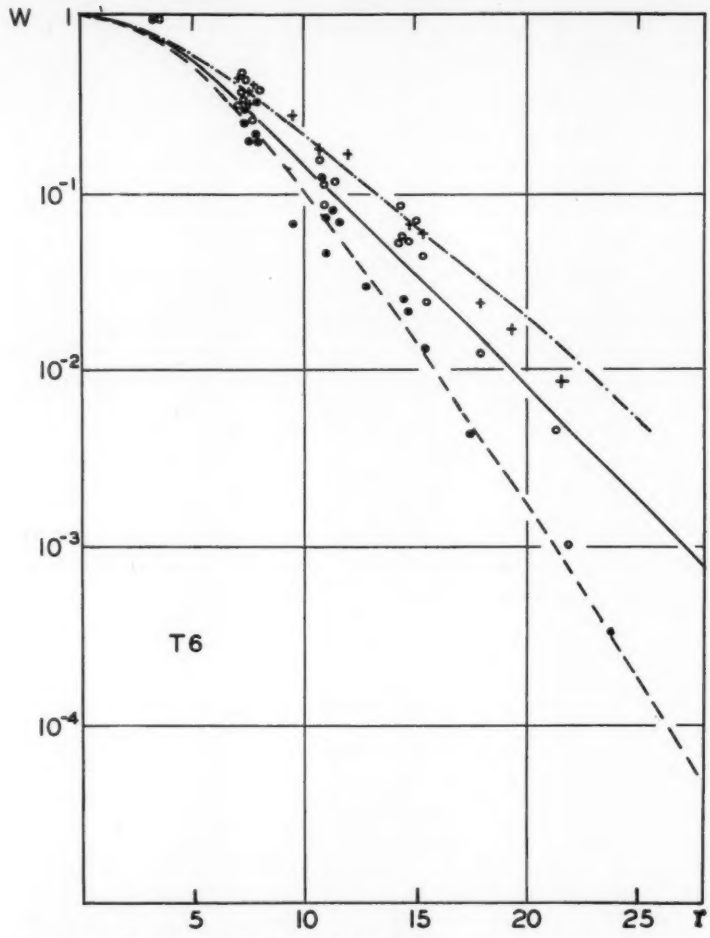

FIGURE 7.—Same as figure 5, for phages *T6* and *T6r*.

● values of w for x=0.1–0.2.
○ values of w for x=0.8–1.5.
+ values of w for x=2.5–4.0.

Broken line: curve of y for n=30, x=0.15. Solid line: curve of y for n=30, x=0.5. Broken and dotted line: curve of y for n=30, x=0.9.

increasing multiplicities is less evident for phages *T2* than for *T6*, and still less for *T4*, diminishing in the same order as the calculated number of units.

To summarize, the results show that the probability of production of active phage from inactive particles depends on the dose of radiation and on the multiplicity of infection in a way similar to the one predicted by the simple theory, which assumes lethal mutations in discrete transferable units of equal radiation sensitivity and a hundred percent efficient recombination of active units to reconstitute active particles. All deviations can be accounted for by a limitation in the efficiency of recombination when the active units derive from more than two inactive particles.

Several explanations may be offered for this limitation; some of them have been tested experimentally. Lysis from without—failure to liberate phage due to excessive multiplicity of infection (DELBRÜCK 1940)—was found not to take place for multiplicities of the order of those for which the deviations from theory occur. Limitations in the number of particles of a given phage that can participate in phage growth were looked for and found (see DULBECCO 1949a), but their magnitude cannot account for the differences between w and y.

The process of reactivation must involve complex mechanisms of transfer of genetic material among phage particles. Whatever these mechanisms, it is reasonable to expect that they will work less efficiently as the number of phage particles increases. Limitations may conceivably be caused by steric reasons—shape of the particles, position in the bacterium—or by physiological reasons—limited number of units of some catalyst, competition for substrates.

Estimation of the number of units

We have compared our results with the theoretical curves for y calculated for different values of n, the unknown number of transferable units per particle. The curves that best fit the results for phages *T2* and *T2r* are those for $n=25$; for *T6* and *T6r*, $n=30$; for *T4* and *T4r*, $n=15$. For phage *T5*, no accurate estimate of n was obtained, but n appears to be lower than for *T4*.

If the interpretation of the results based on the simple theory is justified, we must consider the values thus obtained for n as minimum estimates of the number of radiation-sensitive, transferable units per phage particle. The estimates are minima because of the assumption of equal sensitivity of all units. Should there be units more sensitive than others, they would be hit more often, and in order to obtain the correct probability of reactivation we should assume more units of the less sensitive type. For example, if one unit were twice as sensitive as the average of the others, one locus of the average sensitivity should be added to our estimate in order to distribute the probability of inactivation over all units in such a way that the reactivation probability remains the same.

In our preliminary report (LURIA 1947) we calculated n in a different manner, by assuming that for low multiplicities and low doses, where the probability of reactivation appeared to be approximately constant as a function of x, we could consider all multiple-infected bacteria as double-infected. This corre-

sponded to putting $k=2$ in formula (6) and to comparing the experimental values of w with the values of y for $x=0$. Upon closer analysis, this method proved incorrect, because the contribution of higher multiple infection cannot be neglected, even for low multiplicities. Analysis of more data showed that the probability of reactivation is in fact not constant for low values of x, but appears to be so for low doses, because the differences are small and of the order of the experimental errors. The use of the wrong approximation made our previous estimates of n too high.

Yield of active phage from bacteria in which reactivation occurs

The yield of active phage following reactivation was studied systematically for phages *T2r* and *T4*. After infection, bacteria were diluted and allowed to lyse in liquid, as in a typical "one-step growth" experiment. The latent period before lysis is somewhat longer than for active phage (about 26 minutes instead of 21 for *T2*, 30 minutes instead of 25 for *T4*), and the rise in phage titer upon liberation somewhat slower. All yields were calculated from plaque counts after the titer had reached a steady level. The results, shown in table 6, indicate that the yields are generally somewhat lower than those from bacteria infected with active phage particles. No clear relation of yield to dose of radiation or to probability of reactivation was detected. For *T2r* irradiated with high doses, there is a certain tendency toward higher yields for higher multiplicities.

Mixed infection with active and inactive phage

Transfer of genetic material involved in reactivation must occur between active and inactive phage particles, since, as we saw before, an active particle of a *T-even* phage can reactivate an inactive particle of another *T-even* phage. If transfer occurred by reciprocal exchanges of genetic material, we should expect that upon mixed infection with active and inactive particles of the same phage some of the active particles would receive inactive units and, therefore, be inactivated. This possibility was tested for phages *T2* and *T4* by experiments of the following type.

Bacteria are added to mixtures containing various proportions of active phage and of phage of the same strain irradiated with different doses. For each mixture, the average numbers of active and of inactive particles adsorbed per bacterium are calculated and, hence, the number of bacteria receiving both active and inactive phage. A plaque count before lysis gives the number of bacteria that liberate phage, while a plaque count after lysis gives the yield of phage per bacterium. In this manner, we can determine whether inactive phage suppresses production of active phage from bacteria that also adsorb an active particle, or possibly affects the yield.

The results of these tests can be listed as follows:

(a) a bacterium receiving an active particle plus one inactive particle of the same phage—no matter how many hits the latter has received—never fails to liberate active phage;

(b) part of the bacteria that adsorb one active particle plus several inactive

ones (the latter carrying enough lethal hits, so that reactivation does not occur among them) fail to liberate active phage. This suppression of active phage production is evident for multiplicities 5 or higher of inactive phage *T4*, and for multiplicities 8 or higher of inactive phage *T2*. The suppression is independent of the time allowed for adsorption of the phages. It may in part be

TABLE 6

The yield of active phage from bacteria in which reactivation takes place

PHAGE	MULTIPLICITY OF INFECTION	DOSE			
		HITS	0	6	12	18
	0.2–0.25		114	51	122	
	0.5–0.6		50	42	22	
	2.9		89		100	29
T2r	7.7		59			60
	9		100	70	94	
	10–11		73	120	59	130
		HITS	0	7.5	15	18
	0.65		260	120	87	200
	1.1		200	87	162	
T4	1.6		275		140	140
	2.7		212		197	
	7–8				135	200
	14–15				250	85

accounted for by the limitation phenomenon described by the junior author (DULBECCO 1949a);

(c) for those bacteria that liberate active phage, the yield per bacterium is not affected by the presence of inactive particles, but remains the same as in controls without the inactive phage;

(d) when bacteria are infected with several heavily irradiated particles that do not give reactivation, and a few minutes later with one active particle, suppression of phage production occurs in a proportion of bacteria that increases with the interval between infections. Suppression is evident with an interval of 2.5 to 4 minutes and practically complete after 10 minutes. These time intervals between infections are, of course, averages, since infection may occur earlier or later for individual bacteria in the same mixture. The yield from those bacteria that liberate phage still remains normal.

The suppression of active phage reproduction by inactive phage in excess indicates the existence of some type of "mutual exclusion" between particles of the same phage. Such exclusion is also indicated by the experiments of DULBECCO (1949a).

More detailed analysis of the interaction between active and inactive phage, using genetic markers, will be reported in future papers. Our results

discussed above are in agreement with earlier observations (LURIA and DELBRÜCK 1942) on interference by a large excess of irradiated phage *T2* with the growth of active phage *T2* when the inactive phage was mixed with bacteria one minute and a half before the active one.

Cross-reactivation between phage particles differing by one character

Only one group of experiments will be discussed here, because of its bearing on the analysis of the data presented in this article. Bacteria were infected with

TABLE 7

Cross-reactivation between one inactive particle of phage T2 and one inactive particle of phage T2r

Compare column (3) with column (1)

EXPERIMENT NO.	DOSE OF RADIATION, HITS	MULTIPLICITY OF INFECTION FOR *T2*	MULTIPLICITY OF INFECTION FOR *T2r*	(1) FRACTION OF BACTERIA RECEIVING *one* INACTIVE PARTICLE *T2* AND *one* INACTIVE PARTICLE *T2r* AMONG THE BACTERIA THAT LIBERATE ACTIVE PHAGE, CALCULATED	(2)* OTHER BACTERIA THAT COULD GIVE MOTTLED PLAQUES (AS FRACTION OF THE BACTERIA THAT LIBERATE ACTIVE PHAGE), CALCULATED	(3) FRACTION OF MOTTLED PLAQUES, FOUND
1A	2.9	0.055	0.055	0.19	0.045	0.14
2A	4.6	0.055	0.049	0.335	0.053	0.16
3A	6.2	0.055	0.055	0.42	0.074	0.155
4A	3.4	0.053	0.053	0.24	0.046	0.14
5A	5.0	0.053	0.053	0.355	0.068	0.20
7A	3.9	0.05	0.05	0.28	0.046	0.14
8A	6.5	0.05	0.05	0.19	0.087	0.21

* The values in this column include all bacteria with two or more particles of one type and one or more of the other type, plus all bacteria with an active particle of one type and an inactive particle of the other. The values are upper limits, since only a fraction of these bacteria will actually give mottled plaques.

phages *T2* and *T2r*, both irradiated ($r = 5$ or 6). Low multiplicities were used, so that a large proportion of the infected bacteria only received one inactive particle, and, of those that received two, a great proportion received one particle of each type. The infected bacteria were plated before lysis, and the plaques examined for the proportion of "mottled plaques," that is, of plaques containing both *T2* and *T2r* active phages. Such plaques can only arise from bacteria infected with both phages. It is seen from the data shown in table 7 that more than half the bacteria infected with one inactive particle of each of the two phages actually liberate a mixture of active particles of both types.

This proves that recombination cannot result from reciprocal exchanges of

units between the infecting particles bringing together into one particle all the active units before multiplication begins. Evidently, this particle should be either *T2* or *T2r*, and could not give rise to active particles of both types. This conclusion will be analyzed further in the discussion. Quantitative analysis of the number and contents of mixed yields from mixed infection with *T2* and *T2r* shall be the subject of future publications.

Phages inactivated by X-rays or nitrogen mustard

In a preliminary article (LURIA 1947) it was stated that no reactivation had been detected for *T-even* phages inactivated by hard X-rays, using the same technique employed for ultraviolet. The same was found in our laboratory by MISS M. E. WILLIS for phages *T2* and *T6* inactivated by a nitrogen mustard (methyl bis (β-chloroethyl) amine hydrochloride).

Experiments by MR. J. WATSON, still in progress in our laboratory, have recently shown, however, that phage *T2* inactivated by hard X-rays can take part in reactivation, but this reactivation occurs with such a low probability that special techniques are required for its detection. In part, the low probability of reactivation of X-ray inactivated phage is due to reduced rate of adsorption. This work will be reported by Mr. WATSON in a future publication.

It seems possible that phages *T1* and *T7*, for which no reactivation was detected after ultraviolet inactivation, may also be found by similar techniques to give some reactivation. It is clear that the probability of reactivation will be low if the number of transferable units is small, or if each lethal mutation involves several units. Its detection will be difficult whenever the number of bacteria in which reactivation occurs is small in comparison with the number of bacteria that receive residual active phage.[4]

DISCUSSION

The experiments described above have given results consistent with the hypothesis that inactivation of several and possibly all bacteriophages by ultraviolet light is to be attributed to lethal mutations in discrete units of genetic material. A genetic basis for inactivation of viruses and bacteria by radiation has often been postulated either on statistical grounds or by analogy (see RAHN 1929; LEA 1947). Our results bring new support to this view, and suggest that most, if not all, the inactivating effect of ultraviolet light (2537 Å) on certain phages is due to the production of localized lethal mutations.

The hypothesis of inactivation by lethal mutations and reactivation by transfer of genetic material following multiple infection, as developed in this paper, has been useful in suggesting a quantitative analysis of the reactivation phenomena and has led to fairly accurate predictions of the experimental results. The following discussion assumes the correctness of this working hypothesis.

[4] While this paper was in press, the senior author found that some reactivation by multiple infection takes place with phage *T1* inactivated by ultraviolet light. For equal multiplicity of infection and equal number of hits, the frequency of reactivation is much lower with *T1* than with any of the *T-even* phages and with *T5*. Assuming that the type of analysis presented in this paper applies to the results with *T1*, a value of n smaller than 5 would be obtained.

According to our analysis, active phage is reconstituted from inactive by reincorporation of active units derived, directly or indirectly, from the inactive particles in a kind of hybridization. As in hybridization, the possibility of recombinations between particles of different wild-type phages suggests the existence of common genetic determinants and the absence of complete incompatibility. It is possible that various interference phenomena among different phages may result from such an incompatibility.

Reassembly of material from inactive particles into active ones is a remarkably efficient process. Genetic material from several inactive particles may be brought together, although the relative efficiency of cooperation diminishes as the number of particles involved in this pluriparental reproduction increases.

We have given estimates for the minimum number of transferable units per particle for several phages. It is interesting to notice that phage *T4*, more resistant than the related phages *T2* and *T6* to ultraviolet light (figure 1), appears to have fewer units. This may indicate absence of a portion of genetic material present in the other *T-even* phages.

LEA and SALAMAN (1946), analyzing the dependence of the rate of inactivation of phages by X-rays as a function of the density of ionization, and assuming that the radiosensitive material consisted of spherical units, arrived at the conclusion that a large phage contained 14 such units, whereas a small phage contained one only. Although the hypotheses involved were probably oversimplifications, the conclusion receives qualitative support from our results.

We must consider next the possible mechanisms of genetic transfer. We may divide the mechanisms into two groups, those in which the reproducing element is supposed to be at all times the phage particle as a whole, and those in which the reproducing elements are assumed to be component parts of the particle.

The simplest hypothesis of the first group would be that reactivation results from pairing (or grouping) or the initial infecting particles, followed by reciprocal exchanges such as occur in chromosomal crossing-over; if these exchanges lead to formation of an active particle, the latter proceeds to multiply. This simple hypothesis can easily be disproved. The high efficiency of reactivation would require very large numbers of successive reciprocal exchanges to bring together all active genetic material before multiplication takes place. Mixed infection with one active and one inactive particle should also lead to the occasional loss of active phage, since we know that genetic recombinations occur between active and inactive particles. Finally, the fact that infection with one particle each of inactive *T2* and inactive *T2r* yields a mixture of active *T2* and active *T2r* disproves this hypothesis, since any number of reciprocal exchanges between the original particles *before multiplication* could never lead to formation of active particles of both types.

Another interpretation based on reciprocal exchanges would be that inactive particles reproduce, and that exchanges occur at various stages of the reproduction among the original particles or their inactive offspring. These exchanges should be numerous enough to make the probability of incorporation of all active units into an active particle close to unity, and an ac-

tive particle, once formed, should be favored in multiplication. Without completely disproving it, our results make this explanation very unlikely. The fact that the probability of reactivation increases greatly by increasing (for example, from 8 to 20) the multiplicity of infection with heavily irradiated particles would require a very high number of exchanges. To obtain high yields of active phage in these cases we should assume, moreover, that the exchanges take place early, and that the active particles, once formed, multiply with little or no interference from the inactive particles in large excess. This seems contradicted by the fact already mentioned that active particles can actually undergo interchanges with inactive ones. Altogether, there is strong evidence that inactive units have less chance than active units of entering the final particles, even when the active units derive from inactive particles.

In search for a mechanism that could selectively bring together the active units, the senior author (LURIA 1947) suggested the hypothesis of independent reproduction of individual units to form a "gene pool," from which the new active particles could be derived. Inactive units were considered to be those that cannot reproduce and that have, therefore, little chance of incorporation into the final particles. The tendency to reduction in yield may be due to occasional incorporation of some of the original inactive units. No hypothesis is made as to how the units reproduce or reassemble. The last step is the most difficult to visualize, and we incline to the belief that the original particles may play a role in it, possibly by supplying a framework for reassembly. This is suggested by the limited efficiency of collaboration among large groups of particles, which indicates a certain tendency of the units to remain together with their original companions.

One may ask whether, inside bacteria in which reactivation does not take place, the active units present in the infecting particles reproduce or not. COHEN (1948) states that no desoxyribose nucleic acid is synthesized in bacteria infected with particles of *T2* exposed to doses of ultraviolet light much higher than those employed in our study. Preliminary cytological evidence, collected with the collaboration of DR. C. F. ROBINOW in our laboratory, indicates that in infected bacteria, in which reactivation does not take place, there is no accumulation of stainable material supposedly representing desoxyribose nucleotides. If this evidence is confirmed and found to apply to the conditions of our experiments, it might then suggest, either that there is no reproduction of active units when they are not all present (which might altogether invalidate the hypothesis of independent reproduction), or that at least part of the reproduction of the active units may take place without increase in desoxyribose nucleotides.

The hypothesis of a "gene pool," although by no means the only possible one,[5] fits all results of reactivation. Its validity may soon be amenable to

[5] Another possibility, suggested by DR. A. H. STURTEVANT, would be a process of zipperwise replication of the various units of a phage particle. When in this process an inactive unit was reached, replication could only continue if the partial replica came in contact with another phage particle in which that unit was active. The process would then continue by addition of replicas of the active units of the second phage particle. If repeated several times, such a mechanism would provide for selective recombination of all active units.

critical test. In postulating a phase in phage growth in which particles, as we know them in the extracellular phase, are not present, the hypothesis accounts for the repeated failures to obtain active phage by premature artificial disruption of infected bacteria. It agrees with the observation made by FOSTER (1948) in our laboratory that in the presence of proflavine the reactions leading to production of active phage proceed normally for a part of the latent period, but, upon lysis, no active particle is liberated. According to FOSTER (1948) active phage particles are present in the infected bacterium after the proflavine sensitive stage is passed—beginning 12 to 14 minutes after infection for phages *T2* or *T6*. A similar conclusion was reached by A. H. DOERMANN on the basis of experiments on the effect of other inhibitors on the growth of *T3* and *T4r* (DOERMANN 1948). That viruses multiplying inside the host cell may not have the same organization as in the extracellular form has been suggested before for certain animal viruses (see BLAND and ROBINOW 1939).

Our theory does not assume any degree of linkage among units, although linkage may be compatible with the theory. Each group of strongly linked units would behave as one unit, possibly as a particularly sensitive one. Weakly linked units would probably reduce the probability of reactivation for high doses, when lethal units present in one group would hinder the utilization of the linked active units for reactivation.

In their work on the *r* and *h* mutants of phage *T2 H*, HERSHEY and ROTMAN (1948, 1949) found evidence for a series of determinants exhibiting various degrees of linkage, from those apparently unlinked (high frequency of independent transfer, no correlation between the frequencies of complementary recombinant types in the yield) to others rather strongly linked. For the latter ones, the authors considered that their results suggested the possibility of reciprocal exchanges.

As a working hypothesis we may assume, together with HERSHEY and ROTMAN (1949) that unlinked determinants may be located in different reactivation units, possibly transferred by a gene pool mechanism, while linked determinants may be located in the same reactivation unit. If reciprocal exchanges were found to occur, then homologous units or groups of units should be supposed to pair or group together at some stage in the growth process. Techniques recently developed in our laboratory should soon permit a study of the inactivation of individual genetic determinants and a solution of some of these problems. It appears, therefore, advisable to refrain from further discussion at the present time.

The formation of active phage from inactive by transfer of discrete units requires some revision of the interpretation of experiments on irradiation of phage inside infected bacteria with ultraviolet light (LURIA and LATARJET 1947). In case of multiple infection, the survival curves for phage-producing ability immediately after infection indicated suppression by damage of a number of centers, with the sensitivity of individual centers lower than that of extracellular phage particles. It now seems clear that what was measured was the rate of inactivation of individual units rather than of whole particles. The curves for suppression of the phage-producing ability of multiple-infected

bacteria immediately after infection are similar to the curves for the probability of reactivation for a comparable group of irradiated particles. For example, the suppression curve given by LURIA and LATARJET (1947) for bacteria infected by five particles of phage *T2* is very similar to the curve for the probability of reactivation w as a function of r for $x=5$. It is clear that in interpreting experiments on inactivation of intracellular phage it will be necessary to take into account the occurrence of genetic transfers.[6]

SUMMARY

Coli-bacteriophages *T2*, *T4*, *T5*, and *T6* inactivated by ultraviolet light are still adsorbed by sensitive bacteria. Bacteria infected by only one inactive phage particle are not lysed and do not yield active phage. Infection of bacteria with more than one inactive particle leads to lysis and production of active phage in a fraction of the bacteria. This fraction diminishes with increasing doses of radiation and increases with increasing numbers of particles adsorbed per bacterium. The assumption is made that inactivation is due to lethal mutations in a number of genetic "units" of the phage particle, and that production of active phage from inactive is due to recombination of non-lethal units to form active particles. The values of the probability of active phage production calculated from these assumptions agree with the experimental results with certain limitations. In order to explain the very high frequency of recombination, the hypothesis is proposed that phage growth occurs by independent reproduction of each unit followed by reassembly of the units into complete phage particles. The minimum number of units per particle is estimated for various phages.

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[6] While this paper was in press, one of us (DULBECCO 1949b) discovered that ultraviolet irradiated phages can be reactivated by exposure to visible light of short wave length in presence of bacterial cells. This "photoreactivation" differs from reactivation by multiple infection in many of its features. Photoreactivation does not take place to any appreciable extent under the conditions in which the experiments reported in this paper were performed. A series of experiments of the type exemplified in table 4, but carried out in dim yellow light—under conditions that completely avoid photoreactivation—gave results undistinguishable from those of the earlier experiments, in which no precaution had been taken to control illumination.

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APPENDIX

ON THE RELIABILITY OF THE POISSON DISTRIBUTION AS A DISTRIBUTION OF THE NUMBER OF PHAGE PARTICLES INFECTING INDIVIDUAL BACTERIA IN A POPULATION

R. DULBECCO

In calculating the average number x of phage particles adsorbed per bacterium (multiplicity of infection) from the number of uninfected bacteria, and, from x, the proportion of bacteria with any given number k of particles, the assumption is made that the distribution of particles per bacterium is a Poisson distribution. One limitation to this assumption may arise from differences in the surface area of individual bacteria. This limitation was analyzed as follows.

In a mixture of P particles and B bacteria, each phage particle has a probability $p=c(1/B)$ to be adsorbed by a given bacterial cell. In actual cases where P and B are large and c is between 0.4 and 0.9, we have : $x=cP/B$.

If c is constant for all bacteria, the distribution of k is a Poisson distribution; if not, the distribution of k will be different. We assume, in first approximation, that the adsorption capacity of a bacterium is proportional to its surface, and that the surface is proportional to the length—considering bacteria as cylinders with uniform diameter and negligible end surfaces.

The distribution of bacterial lengths was obtained experimentally on standard cultures of *E. coli* B containing 10^8 cells per ml. Negative stains with

nigrosin were made without fixation, and, after the slides were dried, 764 cells were measured with an ocular micrometer (Filar). The distribution of lengths is given in figure 8, and will be referred to as the "B distribution." Measurements on five cultures gave similar distributions.

For the purpose of obtaining the distribution of phage particles adsorbed per bacterial cell, or "P distribution," we shall split the bacterial population in-

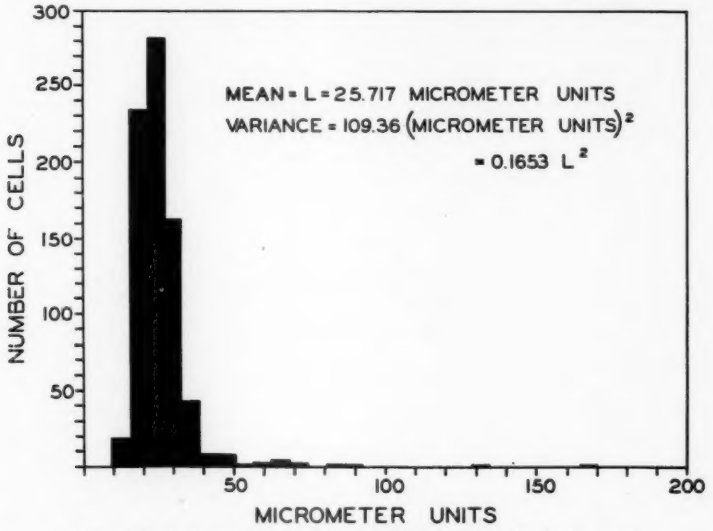

FIGURE 8.—The distribution of bacterial lengths in standard cultures of *Escherichia coli* strain B containing 10^8 cells per ml.

to a number of subpopulations, each comprehending one of the length classes in figure 8, and assume that the adsorption capacity of all cells within each subpopulation is constant.

By the calculation given at the end of this paper, the following properties of the P distribution are derived:

a) its arithmetic mean is equal to x, that is, to the multiplicity of infection, as for a Poisson distribution.

b) its variance is equal to the variance x of the Poisson distribution, plus a term equal to the variance of the B distribution: $\mathrm{var(P)} = x + \mathrm{var(B)}$.

If we express the variance in terms of the mean x taken as the unit of measure, we obtain for our experimental cultures: $\mathrm{var(P)} = x + 0.1653x^2$. This equation expresses the relation between the variance of the P distribution for an ideal population with uniform capacity of adsorption and the variance for a real population with a capacity of adsorption distributed with a given variance.

The P distributions for several values of x, calculated from the actual B distribution, are given in table 8, together with the Poisson distributions

for the same values of x. It is clear that for low multiplicities of infection there is little difference between actual and Poisson distributions. For higher multiplicities, the discrepancies increase; still, for $x=1.5$, they only affect relevantly the proportions of bacteria with 5 or more particles.

TABLE 8

The P distribution for various values of x

"P" columns give the frequencies in the P distribution, "Poisson" columns give the frequencies in the Poisson distribution. All frequencies are multiplied by 10^6.

K	x=0.2		x=1.5		x=2.5		x=5		x=7.5		x=10	
	P	POISSON	P	POISSON	P	POISSON	P	POISSON	P	POISSON	P	POISSON
0	82115	81873	24686	22313	10400	8208	1409	674	228	55	32	5
1	15977	16374	32918	33468							218	45
2	1735	1637	23197	25101							765	227
3	155	109	11658	12551							1908	757
4	14	6	4751	4707							3739	1892
5	2		1721	1412							6065	3784
6			611	353							8411	6303
7			238	76							10218	9009
8			109	14							11081	11261
9			62	2							10924	12512
10			38								9942	12512
11			29								8489	11375
12			19								6902	9479
13			12								5383	7292
14			8								4089	5208
15			5								3036	3472
16			3								2219	2170
17			1								1600	1276
18			1								1140	709
19											810	373
20											579	187
21–23											931	147
24–26											464	11
27–30											293	
31–33											172	
34–36											110	
37–39											67	
>39											305	

The calculation of x from the bacterial survival—assuming (B uninfected)/$B=e^{-x}$—only gives reliable results for values of x up to about 2. For higher multiplicities, it leads to an underestimation of x.

THEORY

The bacterial population is considered as a mixture of an infinite number of subpopulations homogeneous in adsorption capacity. Within each subpopulation there is a Poisson distribution of phage particles per bacterium, whose frequency function is:

$$f_j(k) = \frac{e^{-x_j} x_j^{\,k}}{k!} \qquad (1)$$

where x_j is the multiplicity of infection in the jth subpopulation. x_j is obtained from the B distribution of bacterial lengths: if we call l_j the length of the bacteria in the jth class, we have:

$$x_j = x\frac{l_j}{L} \tag{2}$$

where x = multiplicity of infection in the total population, L = arithmetic mean of the B distribution (average bacterial length).

The frequency function of the distribution of phage particles per bacterium in the *total* population (P distribution) is

$$f(k) = \sum_{j=0}^{\infty} n_j f_j(k) = \sum_{j=0}^{\infty} \frac{n_j e^{-x_j} x_j^{\,k}}{k!} \tag{3}$$

where n_j is the frequency of the jth subpopulation in the B distribution

$$\sum_{j=0}^{\infty} n_j = 1$$

The Poisson distribution of phage particles per bacterium within each homogeneous subpopulation of bacteria has the following first two moments:

First moment about the origin $= (\mu_1')_j = x_j$

Second moment about the mean $= (\mu_2)_j = x_j$

For the P distribution in the total population, remembering formula (3), we have a mean

$$(\mu_1')_P = \sum_{k=0}^{\infty} k \sum_{j=0}^{\infty} n_j f_j(k) = \sum_{j=0}^{\infty} n_j \sum_{k=0}^{\infty} k f_j(k) = \sum_{j=0}^{\infty} n_j (\mu_1')_j = \sum_{j=0}^{\infty} n_j x_j;$$

from formula (2) we obtain:

$$(\mu_1')_P = x. \tag{4}$$

To find the variance $(\mu_2)_P$ of the P distribution, let us remember that, in general,

$$\mu_2 = \mu_2' - (\mu_1')^2 \tag{5}$$

where μ_2' is the second moment about the origin. We have then:

$$(\mu_2')_P = \sum_{k=0}^{\infty} k^2 \sum_{j=0}^{\infty} n_j f_j(k) = \sum_{j=0}^{\infty} n_j \sum_{k=0}^{\infty} k^2 f_j(k) = \sum_{j=0}^{\infty} n_j (\mu_2')_j$$

where $(\mu_2')_j$ is the second moment about the origin of the distribution of phage particles per bacterium in the jth subpopulation. But $(\mu_2')_j = x_j + (x_j)^2$ (since, in general, $\mu_2' = \mu_2 + (\mu_1')^2$), and, for each homogeneous subpopulation, $(\mu_2)_j = (\mu_1')_j = x_j$. We obtain, therefore:

$$(\mu_2')_P = \sum_{j=0}^{\infty} n_j (\mu_2')_j = \sum_{j=0}^{\infty} n_j x_j + \sum_{j=0}^{\infty} n_j (x_j)^2. \tag{6}$$

Finally, introducing the values obtained from (4) and (6) in formula (5):

$$(\mu_2)_P = \sum_{j=0}^{\infty} n_j x_j + \sum_{j=0}^{\infty} n_j (x_j)^2 - x^2. \tag{7}$$

The first term on the right side of equation (7) equals x; the other two terms equal the variance of the B distribution, expressed as function of x. We obtain therefore,

$$(\mu_2)_P = x + \text{var (B)}.$$

THE NUMBER OF PARTICLES OF BACTERIOPHAGE T2 THAT CAN PARTICIPATE IN INTRACELLULAR GROWTH[1]

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HERSHEY (1946) and LURIA and LATARJET (1947) have shown that more than one particle of the same phage type can participate in the process of intracellular growth inside the same bacterial cell. The question arises whether there is any limitation to the number of particles that can take part in this process (LURIA and DULBECCO 1949). An indirect approach to this question was attempted as follows.

THEORY

Let us consider a population of bacterial cells simultaneously infected with particles of the two phages *T2r*$^+$ and *T2r*.

Supposing that for each phage the number of particles adsorbed by every bacterium is the same, for example, 20 particles for *T2r*$^+$ and one for *T2r*, the yield from these cells will depend on whether all particles or only a limited number participate in the process of multiplication. In the case of unlimited participation, we shall find both *r* and *r*$^+$ particles in the yield of each bacterium. In the case of limited participation, *r* particles will fail to be produced in a fraction of bacteria that can be calculated easily by assuming that there is no advantage for either type of virus in the course of growth.

Let us assume that a bacterial cell adsorbs m particles of phage A and n of phage B, and that only K particles can participate in growth. The number of possible groups of K particles is given by $\binom{m+n}{K}$. The number of groups containing no A particles is $\binom{n}{K}$. The probability P (A=0) of obtaining yields without phage A is

$$P(A = 0) = \binom{n}{K} \Big/ \binom{m+n}{K} = \frac{n^{[K]}}{(m+n)^{[K]}} = \frac{n!(m+n-K)!}{(m+n)!(n-K)!} \qquad (1)$$

where: $n^{[K]} = n(n-1) \cdots (n-K+1)$. Similarly, the probability P(B=0) of obtaining a yield without B is:

$$P(B = 0) = \binom{m}{K} \Big/ \binom{m+n}{K} = \frac{m^{[K]}}{(m+n)^{[K]}} = \frac{m!(m+n-K)!}{(m+n)!(n-K)!}.$$

The total probability of having unmixed yields is expressed by:

[1] This work was done under an AMERICAN CANCER SOCIETY grant, recommended by the COMMITTEE ON GROWTH OF THE NATIONAL RESEARCH COUNCIL, and under the direction of DR. S. E. LURIA.

$$P(A \text{ or } B = 0) = P(A = 0) + P(B = 0)$$

$$= \binom{n}{K} \Big/ \binom{m+n}{K} + \binom{m}{K} \Big/ \binom{m+n}{K}$$

$$= \frac{m^{[K]} + n^{[K]}}{(m+n)^{[K]}}.$$

In actual populations, the numbers of phage particles adsorbed by individual bacteria vary over a wide range around an average value x ("multiplicity of infection"). The distribution of the numbers adsorbed by the individual bacteria will be considered in first approximation as a Poisson distribution.[2] The proportion of bacteria that adsorb m particles of A is

$$f(m) = e^{-x_A} \frac{{x_A}^m}{m!}$$

where x_A is the multiplicity of phage A in the whole population of cells. The same bacteria also adsorb phage B. Since the total amount of adsorbed particles is below the maximum adsorption capacity, the distribution of B is independent of the distribution of A. If x_B is the multiplicity for phage B, the proportion of cells that adsorb n particles of B is:

$$f(n) = e^{-x_B} \frac{{x_B}^n}{n!}.$$

The proportion of cells that adsorb m particles of A *and* n of B is:

$$f(m, n) = e^{-x_A} \frac{{x_A}^m}{m!} e^{-x_B} \frac{{x_B}^n}{n!}.$$

The product of $f(m,n)$ by the probability P(A or B=0) of having unmixed yield gives the proportion of bacteria with unmixed yield among those bacteria that adsorb m particles of A and n particles of B. To obtain the proportion P_0 of bacteria that give unmixed yield in the total population, we must sum the products $f(m,n) \times P(A \text{ or } B=0)$ for all values of m and n greater than zero.

$$P_0 = \sum_{m,n>0} e^{-x_A} \frac{{x_A}^m}{m!} e^{-x_B} \frac{{x_B}^n}{n!} \quad \frac{m^{[K]} + n^{[K]}}{(m+n)^{[K]}}.$$

We do not include in the sum the classes of bacteria with $m=0$ or $n=0$, since they cannot give mixed yields. P_0 is, therefore, the probability of ob-

[2] The actual distribution of phage particles per bacterium (DULBECCO 1949) differs increasingly from a Poisson distribution as the x increases. The use of a Poisson distribution in calculating P_0 might, therefore, lead to important errors. To test this point, we calculated P_0 using the distributions of particles per bacterium given by DULBECCO (1949, table 8) for $x_A = 1.5$, $x_B = 10$. The value calculated in this way is about 10 percent lower than the one obtained using the Poisson distribution. This correction would tend to make the value of K lower by an amount that hardly affects either the shape of the curves in figure 1 or the estimate of K given below.

taining, as a consequence of the limitation, an unmixed yield from those bacterial cells that would give a mixed yield in the absence of limitation, or, in other words, *the relative loss of mixed yields as a consequence of the limitation.* Such quantity can easily be compared with experimental data. P_0 was calculated for $x_A = 1$, 1.5, 2 and for x_B between 1.5 and 20; the curves for P_0 (as a function of x_B for $x_A = 1.5$) are reproduced in figure 1[2].

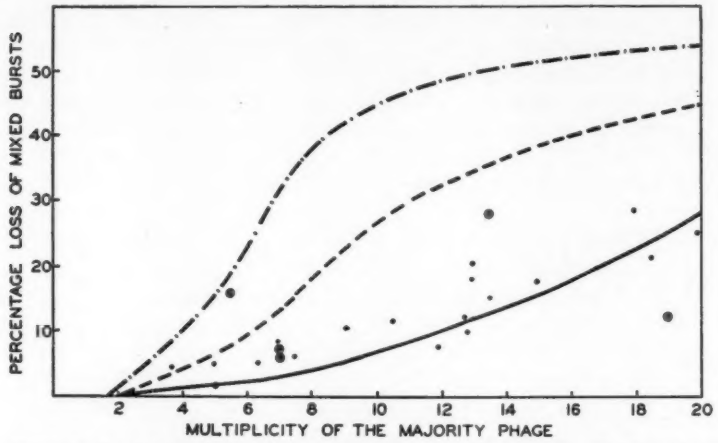

FIGURE 1. Loss of mixed yields in mixed infection with *T2r*$^+$ and *T2r*. The lines give the calculated loss for a multiplicity 1.5 of the minority phage and for $K = 10$ (solid line), $K = 6$ (broken line), or $K = 4$ (broken and dotted line). • Loss found in mottled plaque experiments. ⊙ Loss found in single burst experiments (not controlled by mottled plaques). The minority phage was used with multiplicities between 0.94 and 2.04.

EXPERIMENTAL

Samples of cultures of *E. coli B* containing 10^8 cells per ml were mixed with phages *T2r*$^+$ and *T2r* in amounts that gave a constant multiplicity of infection (between 1 and 2) of one phage and various multiplicities of the other. After six minutes, each mixture was diluted in high titer anti-*T2* serum to stop adsorption and neutralize the unadsorbed phage (DELBRÜCK 1945b.). Five minutes later, further dilutions were made. One sample was incubated for 80 minutes and assayed to determine the average composition of the yield of phage from each mixture. The proportion of mixed bursts was determined in the two following ways:

1. In all experiments, samples were plated before lysis, and the type of plaque produced by each infected bacterium was examined after 8 hours of incubation at 37°C. At this time, the phages give well developed types. If a bacterium gives a mixed yield of *r* and *r*$^+$ particles, the plaque is clearly mottled (HERSHEY 1946).

The mottled plaques are recognizable with great precision when the *r*$^+$ type is predominant in the yield, but some of them may be missed if the pro-

portion of r^+ particles in the yield is small, because in this case the plaques often appear as pure r plaques. In the latter case the counting of mottled plaques was replaced by the counting of mixed yields, as described in the next paragraph.

2. In some experiments the technique of single bursts was used (DELBRÜCK 1945a). The suspension of infected bacteria was divided before lysis began into a number of test tubes (generally from 60 to 100 for every mixture) in such a way that each test tube contained 0.7 bacteria on the average. The bacteria were allowed to lyse in the test tubes at 37°C, and afterwards the whole contents of the test tubes were plated on nutrient agar, so that it was possible to study directly the composition of each yield. Under these conditions, about 50 percent of the plates are fertile ($e^{-0.7} = 0.497$). About 25 percent of the fertile plates contain more than one bacterium but the numbers of mixed and unmixed bursts can be obtained by simple calculations.

The single burst method and the mottled plaque method were compared in several experiments with phage r^+ in excess, in which both techniques were used. The percentage values of mixed bursts obtained by the two methods differ at most by ten percentage units. This can be considered a very satisfactory agreement, in view of the large statistical fluctuations to which both methods are subject. The results, shown in table 1, show that a loss of mixed bursts occurs in every mixture with one phage type in excess over the other, and that the amount of loss is correlated with the multiplicity of the phage in excess, if the multiplicity of the other phage is constant.

According to the theory, no loss should be expected when the multiplicity of both phages is low and equal; among 9 experiments in which this point was tested, 4 cases showed no loss; 3 cases showed a loss of 2–5 percent; in 2 cases the loss reached 10.5 and 14 percent respectively. This loss is probably due to the approximation inherent in the techniques used, since some of the mixed bursts may occasionally be missed.

To obtain comparable data from all experiments, the value of the apparent loss of mixed bursts in the mixture with equal low multiplicities—which was included in almost every experiment—was substracted from the results of every point (column of "corrected loss" in table 1). The values obtained in this way are plotted in figure 1 and follow with good approximation the theoretical curve for a value of K between 8 and 10; the data show rather high fluctuations, to be expected in this kind of analysis, particularly for values obtained by single burst experiments.

Table 1 also shows that the average ratio r/r^+ in the yield from each mixture is approximately equal to the average ratio r/r^+ in the input, no matter which type is prevalent. In single burst experiments we observe that in the yield of the bacteria that give both phages the ratio between the two types is shifted in favor of the minority type. This is due to the fact that the phenomenon of quantitative limitation acts in the same way on particles of either type. The gain of minority phage in the bacteria that give both phages is due to elimination of particles of the majority phage, and compensates for the loss

TABLE 1

The loss of mixed yields from mixed-infected bacteria as a function of the multiplicity of the phage type present in excess.

EXPERIMENT NO.	MIXTURE NO.	MULTIPLICITY OF *T2*	MULTIPLICITY OF *T2r*	RATIO r^+/r IN INPUT	RATIO r^+/r IN YIELD	MOTTLED PLAQUES FOUND, PERCENT	MIXED BURSTS AMONG SINGLE BURSTS, PERCENT	MAXIMUM MOTTLED PLAQUES EXPECTED, PERCENT	LOSS OF MOTTLED PLAQUES, PERCENT	CORRECTED LOSS OF MOTTLED PLAQUES, PERCENT
4	1	19.5	1.8	10.5		45		83	37	23
	2	12.25	1.8	5.25		62		83	21	7
	3	2.05	1.8	0.9		52.5		66	14	0
6	1	15	1.4	10.7		54		75	21	17.5
	2	7.5	1.4	5.4		65.5		75	9.5	6
	3	3.7	1.4	2.6		65		73	8	4.5
	4	1.2	1.4	0.9		49		52.5	3.5	0
8	1	13	1.22	10.65	10.7	50	50	71	21	21
	2	6.5	1.22	5.3	3.3	63	64	71	8	8
	3	1.1	1.22	0.9	0.75	49	58	48	0	0
9	1	18.2	1.51	12	8.9	61		90	29	29
	2	9.1	1.51	6	4.4	80		90	10	10
	3	1.1	1.51	0.89	0.96	81		78	0	0
11	1	12.8	1.06	12	8.6	54.5		65	9.5	9.5
	2	6.4	1.06	6	7.05	60		65	5	5
	3	1.07	1.06	1	1.17	43		43	0	0
14	1	13.5	2.04	6.66	7.8	61		86.5	25.5	15
	2	27	2.04	13.3	15	45.5		86.5	41	30.5
	3	13.5	1.02	13.3	13.3	43		63.2	20.2	3.7
	4	2.7	2.04	1.33	1.2	71		81.5	10.5	0
16	1	25	1.9	13.3	13	50.5	42	85	34.5	28.5
	2	2.5	1.9	1.33	2.2	72	63	78	6	0
	3	12.5	1.9	6.6	8.4	61		85	24	18
17	1	25.6	1.55	16.5	18.5	35		79	44	29.5
	2	2.56	1.55	1.65	3.4	58		72.5	14.5	0
	3	12.8	1.55	8.25	8.2	52.5		79	26.5	12
20	1	25	1.68	15	13	56	46.5	81	25	25
	2	5	1.68	3	3.8	79	77.5	81	2	2
22	1	17	0.93	18	11	38		60	22	21
	2	17	0.93	18	14	39		60	21	20
	3	3.4	0.93	3.4	2.9	57		58	1	0
24	1	21	1.45	14.5	14.5	49		76.5	27.5	22.5
	2	10.5	1.45	7.3	7.35	60		76.5	16.5	11.5
	3	4.2	1.65	2.9	2.84	65		75	10	5
21	1	2.2	24	0.09	0.09		53	89	36	19
	2	2.2	7	0.31	0.48		65	89	24	7
23	1	2.15	19	0.11	0.11		57	88	31	13
	2	2.15	6	0.36	0.32		63	88	25	7
25	1	1.3	13.5	0.09	0.09		45	73	28	28
	2	1.3	5.5	0.27	0.17		57	73	16	16
	3	1.3	1.1	1.2	1		49	49	0	0
18	1	12	12	1	1.2	99	100	100	1	

of minority phage in the unmixed bursts. This explains the equal ratio of the two types in the input and the mass yield (see also page 132), and indicates that neither of the two types has any selective advantage during intracellular growth (HERSHEY 1946).

Control experiments were carried out to determine the influence of factors other than the quantitative ratio of the two phages in the input:

1. Two experiments performed with short adsorption time (Experiment No. 20, 1.5 minutes, Experiment No. 9, 2.25 minutes) gave results similar to those obtained with six minutes adsorption, showing that the loss of mixed bursts cannot be attributed to some interference dependent on the interval of time between infection with different particles.

2. A possible effect of antiserum on particles that had just been adsorbed was excluded by Experiment No. 22, in which the serum treatment was delayed five minutes after the end of adsorption with no change in the results.

3. Other experiments excluded loss of infective centers by excessive multiplicity (lysis from without, DELBRÜCK 1940) and showed that the results could be reproduced with different stocks of phages *T2r*$^+$ and *T2r*. In Experiment No. 18, in which both phages were used in high multiplicity, practically all plaques are mottled, as expected if there is no loss due to causes other than limitation.

DISCUSSION

The quantitative agreement between experimental data and theory supports the hypothesis that only a limited number of phage particles can participate in the process of multiplication. This maximum number is of the order of 8 to 10. This result may either indicate that a limited number of phage particles can penetrate into a bacterium, or simply be a formal expression of a phenomenon that occurs later in the growth process and results in the apparent limitation. The value thus obtained for K is an average value. The number of particles that take part in growth may actually fluctuate from cell to cell. If phage growth takes place by independent multiplication of genetic "loci," as suggested by LURIA (1947), the limitation that we have described might even only be involved in the growth of the specific locus concerned with the determination of the pair of allelic characters studied. The study of other characters might give different results.

The phenomenon of limitation may be an important source of variability in experiments in which mixed infection of a bacterial population is used with rather high multiplicity. In fact, following the same reasoning that leads to the formula (1), representing the probability that one type of phage is completely excluded, it is possible to determine the probability that any given number of particles of a phage will take part in the process of multiplication in bacteria that also receive particles of another phage.

Using the same notation as before, the probability of having s particles of virus A and $(K-s)$ of virus B participating in the process of multiplication in the bacterial cell is

$$P(s) = \binom{K}{s} \frac{m^{[s]} n^{[K-s]}}{(m+n)^{[K]}} . \qquad (2)$$

Let us put: $N = n+m$ = total number of phage particles adsorbed by a given bacterium; $p = m/(n+m)$ = proportion of A particles adsorbed; $q = n/(n+m)$ = proportion of B particles adsorbed. We find (KENDALL 1944, p. 127) that the mean, μ_1' and the variance μ_2 of the distribution (2) of P (S) for different values of S are:

$$\mu_1' = Kp \qquad \mu_2 = \frac{Kpq(N-K)}{N-1}$$

From the expression of the mean we see the theoretical reason for the fact, found experimentally, that the average ratio of the amounts of the two viruses that undergo multiplication—as judged by the composition of the yield—is equal to the ratio of the adsorbed phages: the latter ratio is $Np/Nq = p/q$, while the corresponding average ratio of the phages that take part in multiplication is $Kp/Kq = p/q$.

From the expression of the variance we deduce that, due to the limitation, the fluctuations in the composition of yield from single bacterial cells are expected to increase rapidly as the multiplicity increases to value above K.

SUMMARY

Mixed infection of sensitive bacteria with particles of phage *T2* and of its mutant *T2r* leads in a certain proportion of cases to the liberation of particles of one type only, if that type was represented in excess among the infecting particles. A theory has been developed to yield predictions as to the frequency of unmixed yields from mixed-infected bacteria, by assuming that only a fixed number K of infecting particles can contribute to the final yield of new particles. The experimental results agree well with the theoretical expectations, and indicate that K has a value around 8 or 10.

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A QUANTITATIVE HISTOLOGICAL STUDY OF THE PIGMENT FOUND IN THE COAT-COLOR MUTANTS OF THE HOUSE MOUSE. III. INTERDEPENDENCE AMONG THE VARIABLE GRANULE ATTRIBUTES[1]

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THERE is considerable reason to believe that the coat-color genes of mammals provide particularly favorable material for the study of the physiology of gene action, as the chain of reactions between gene and final character must be short relative to that in most internal and embryologically determined characteristics (E. S. RUSSELL 1946). Therefore a histological analysis of the pigments found in the hair of the house mouse has been undertaken in an attempt to find as much as possible about the end product of the reaction chain initiated by the action of the pigment genes. The first stage of this study was measurement of variations in the pigment granule attributes which were found to contribute materially to the coat-color differences observed among 36 mutant types tested. Seven such variable attributes were recognized and measured separately for each type: number of pigment granules (1) per medullary cell and (2) per unit volume of cortex, (3) tendency to distal arrangement of pigment within the medullary cells, and the (4) size, (5) shape, (6) clumping, and (7) color of the individual granules (E. S. RUSSELL 1946). After this strictly analytical approach an estimate of the total volume of pigment in each genotype was prepared by combining certain of these measurements with new determinations of individual granule volume (E. S. RUSSELL 1948).

While many facts about the causes of color differences could be determined simply by studying separately the condition of each of the variable attributes in all of the genotypes, it was felt that just as combining information on several separate attributes gave a measure of total pigment volume valuable to further interpretation of gene action, knowledge of other interrelationships among the attributes could provide background necessary for an analysis of the basic action of the genes involved.

In this paper each attribute will be further discussed, and all possible interdependences among them presented in a search for key characters whose variations determine all the rest. Tables and figures will be given of the variations pertinent to each topic discussed, but for a complete picture of all measurements of each genotype and of the methods used, the reader is referred to the earlier papers of the series.

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THE RELATIONSHIP OF GRANULE COLOR TO THE OTHER PIGMENT ATTRIBUTES

There are three color series into which fall the colors of all the pigment granules observed. The simplest is the yellow series, found in the 12 pigmented yellow types, whose pigment should probably be called "phaeomelanin" or "xanthin" (WRIGHT 1941) in contrast to the dark-colored "eumelanin" of the other two color series. The granules of the eight pigmented non-agouti black genotypes are of the second, or black-fuscous series, and those of the six non-agouti browns of the third or brown series. Agouti genotypes contain, in different regions of the hair, granules of the first and of the second or third series.

This qualitative difference in the nature of granule color appears to impose limitations upon certain other granule attributes. The most conspicuous difference associated with color is the quantity (measured by total pigment volume) produced in types with each of the colors. The highest total pigment volume value in a black-fuscous genotype is 1332 in *aaBBCCDDPP*, and four out of eight such genotypes have values over 700. The highest value in a brown is 480, and in a yellow, 281 (table 1). Another attribute whose variation is affected by color is granule number. The upper limit of medullary granule number is the

TABLE 1

The limitations upon other granule attributes imposed by color.

ATTRIBUTE	COLOR SERIES		
	YELLOW	BLACK-FUSCOUS	BROWN
Total pigment volume, highest	281	1332	480
Upper limit, medullary number	47±2	98±2	95±4
Upper limit, cortical number	39±7	219±7	160±8
Color intensity	aniline yellow	black, black-fuscous, fuscous	carob brown mummy brown
Shape	round	long-oval, oval, round, shred	round, shred
Mean greater diameter, μ	0.83–0.66	1.44–0.62	0.98–0.61

same in blacks and browns, but much lower in yellows (table 1). The upper limit of cortical number is different for each series, highest in black-fuscous types, intermediate in browns, lowest in yellows (table 1).

The extent of variation in a number of other attributes seems to be limited by nature of granule color: intensity of color, and size and shape of granules (table 1). All xanthic granules are of approximately the same intensity, the same round shape, and almost the same size. The black-fuscous granules are much more variable in all these characteristics, with three grades of color, four recognizably different shapes, and wide variation in granule size. Brown granules are intermediate in amount of variation, with two grades of color, two shapes, and a range of sizes just slightly larger than that in yellows.

Thus we have shown clearly the interdependence with quality of color of several other granule characteristics, such as total pigment volume, medullary

and cortical granule number, amount of intensity variation, and size and shape of granules. The total pigment volume in black types is much higher than in either brown or yellow. In general the yellow seems very stable in characteristics, the black-fuscous very labile, and the browns intermediate. Although we do not know a great deal at the present time about chemical differences among the three pigments, there certainly are great differences in the types of granules possible in the three series and in their reactions to changes in the genetic background.

EFFECTS ASSOCIATED WITH SIZE OF GRANULES

It has already been shown that the size and size variation of pigment granules differ markedly in the three color series. It would be interesting to see what other granule attributes are closely associated with granule size.

If the nature of the pigment were identical in all granules within a given color series, differences in color intensity of granules should result only from differences in the amount of pigment absorbing light in each granule, which might very well depend upon the size (thickness) of the granule. The small amount of size variation and lack of intensity variation in yellows support this idea, as also does the great variation in both attributes in black-fuscous granules and the intermediate amount in browns (table 1). In the black-fuscous series, the correspondence between intensity and size is particularly striking (figure 1). The largest granules, in *aaBBCCDDPP*, are intense black; those averaging between 1.00 and 1.25 μ greater diameter (*aaBBCCddPP*, *aaBB*$c^{ch}c^{ch}$*ddPP*, and *aaBB*$c^{ch}c^{ch}$*DDPP*) are fuscous black; and all smaller granules, whether spherical or shred-shaped, are of the lighter fuscous shade. In the brown series, the four genotypes with the larger average granule size are all the darker carob brown, while the two smaller-granuled, irregular shred types, are the lighter mummy brown.

However, since crude examination of the data for black-fuscous and brown types suggests that variations in medullary granule number as well as size run somewhat parallel to changes in color intensity, the relation between number and intensity must also be examined. More careful study shows that in the black-fuscous types, correspondence with granule number, while present, is not so perfect as with granule size. For example, in one substitution (*aaBB*$c^{ch}c^{ch}$*DDPP* for *aaBBCCDDPP*) the intensity change from intense black to fuscous-black is accompanied by a change in granule diameter from 1.44 μ to 1.05 μ, while there is no change in granule number. In the brown series there is no exclusive correspondence between color intensity and number of granules per medullary cell: the numbers in carob brown types range from 43 ± 2 to 90 ± 2, and in mummy browns from 41 ± 2 to 49 ± 2.

Combining the three color series, there is still a close relationship between granule size and color intensity (figure 1). As we can find no case where the color intensity of a granule changes without its size also changing, and since the extents of both changes run parallel, color intensity is almost certainly a consequence of granule size.

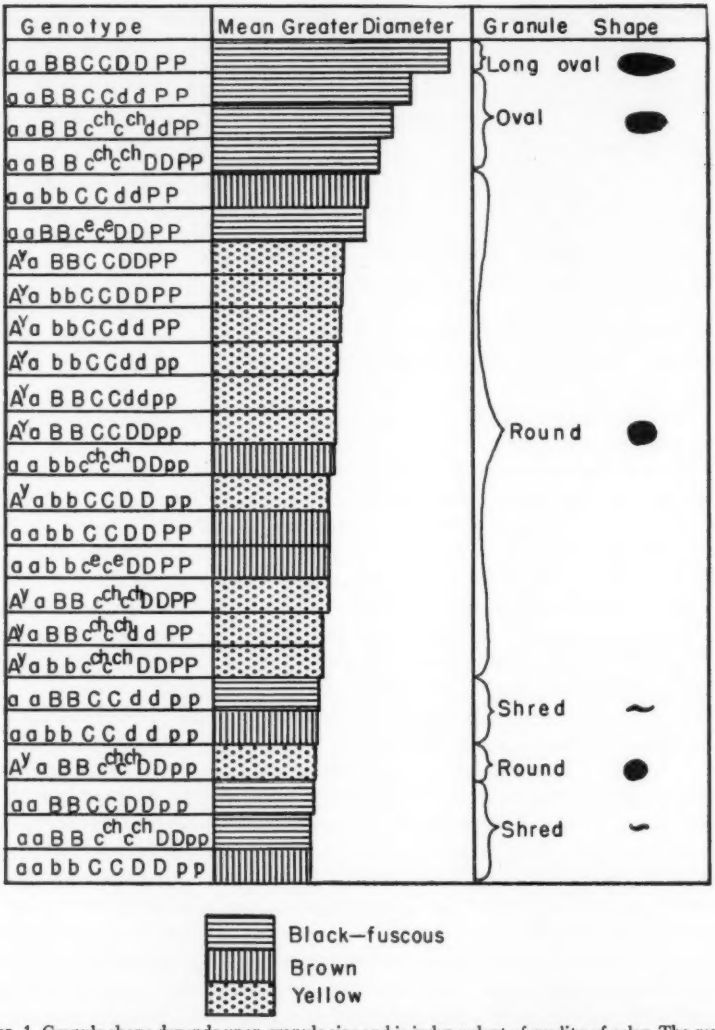

FIG. 1. Granule shape depends upon granule size and is independent of quality of color. The genotypes are arranged in descending order of mean greater diameter, from 1.44 μ down to 0.61 μ.

The interrelation of size and shape of granules is an interesting question, not important in the yellow series, where there is no variation in shape and little in size, not particularly noticeable in browns (although the only shape variation (spheres and irregular shreds) does separate the four larger from the two smaller types), but very impressive in black-fuscous types, where there is a great deal of variation in both shape and size of granules (figure 1). The largest granules (mean, 1.44 μ, in *aaBBCCDDPP*, are long ovals. In the three genotypes with means between 1.00 and 1.25 (*aaBBc^{ch}c^{ch}DDPP*, 1.05 μ, *aaBBCCddPP*, 1.23 μ, and *aaBBc^{ch}c^{ch}ddPP*, 1.12 μ the granules are classified as oval. The slightly smaller granules of one genotype (*aaBBc^{e}c^{e}DDPP*, mean 0.94 μ are classified as round, and the still smaller granules of three genotypes (*aaBBCCddpp*, *aaBBCCDDpp*, and *aaBBc^{ch}c^{ch}DDpp*) ranging from 0.73 μ down to 0.62 μ are irregular shreds. It is very interesting and probably significant that the mean sizes of the round granules in four brown genotypes range from 0.98 μ down to 0.77 μ, corresponding with the blacks of the same shape. Similarly, the mean greater diameters of the shred-shaped granules in the other two brown genotypes (0.61 μ and 0.67 μ correspond with the size of black irregular shreds. In general, the shape and size of yellow granules also fits well with the above situation: all yellow granules are round, and in 11 out of the 12 pigmented yellows their mean greater diameters (0.76 μ to 0.83 μ) fall in the same size range as that for round granules in eumelanotic types (0.77 μ to 0.98 μ). In the one exception (*A^{y}aBBc^{ch}c^{ch}DDpp*) the size (0.66 μ) is in the range of shred-shaped granules.

As in the case of color intensity, there are changes in medullary number, especially in black fuscous types, which run somewhat parallel to changes in granule shape. There are, however, cases where shape is changed without significant change in number, as for example, the change from long oval in *aaBBCCDDPP* to oval in *aaBBc^{ch}c^{ch}DDPP* without significant change in number. Conversely, there are cases where extensive changes in number have no effect on shape. There is great variation in number of granules among the shred-shaped black genotypes and also among the brown genotypes with round granules. There appears to be no regular change in shape which always accompanies a change in number.

There is, however, a definite change in shape which always accompanies a certain change in size. The arrangement in figure 1 of all genotypes tested, regardless of color, in a single column of descending granule length indicates this close relationship very clearly, and demonstrates its independence of quality of color. It seems clear that both shape and intensity of color of granules are almost completely dependent upon granule size, and that the only relationship between granule size and nature of granule color is the limitation upon upper limits of size variation set by the nature of the color.

THE COMPLEX OF INTERDEPENDENT FACTORS DESCRIBED AS "DEGREE OF PIGMENTATION"

A very fortunate circumstance in the analysis of the relationship of the number of granules formed in a given hair cell with the characteristics of those

granules was the finding in certain genotypes of variation in the number of granules per medullary cell along the longitudinal axis of single hairs. There is a gradual rise in the medullary count from the tip toward the base of these hairs, usually with a significant rise between pairs of observations taken at intervals of ten microscopic fields (about 850 μ) at least from the 10th field to the 20th, and occasionally as far down as the interval between the 30th and 40th fields. This tendency to increase shows very clearly in the plots of the medullary numbers at 10-field intervals of all non-agouti genotypes in figure 2.

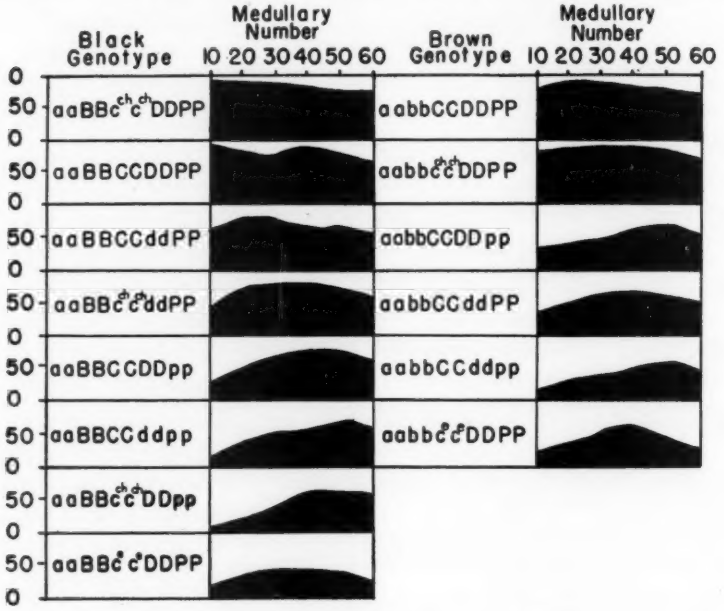

FIG. 2. Demonstration of pigment lag in relation to full medullary number. Non-agouti genotypes are arranged in descending order of full medullary number, with a plot of medullary counts at 10-field intervals for each genotype.

The phenomenon has been termed "pigmentation lag," since the first medullary cells growing out from the matrix cells of the hair follicle in these types fail to produce the full number of granules of which later-appearing medullary cells prove the genotype to be capable. Aside from its intrinsic interest, pigmentation lag is very important in providing a definite case where the amount of pigment produced per medullary cell is changed without a genetic substitution. Such non-genetic changes in the pigmentation reaction may be termed general changes in the degree of pigmentation. Any changes in granule attributes accompanying these changes in degree definitely are not genic effects. Evaluating them will, of course, help to determine the nature of genic effects.

Table 2 shows that six of the 12 yellow genotypes, six of the eight black-fuscous genotypes, and five of the six brown genotypes have significantly

TABLE 2

The ratio of the medullary granule count at the 10th microscopic field to the highest medullary count reached in each of the genotypes tested, arranged in descending order of highest medullary count.

GENOTYPE	MED. COUNT, 10TH FIELD MEAN S.E.	HIGHEST MED. COUNT MEAN S.E.	RATIO 10TH/FULL
aaBB$c^{ch}c^{ch}$DDPP	98±2	98±2	1.00±.03
aaBBCCDDPP	93±3	95±11	.98±.13
aaBBCCddPP	59±5	73±5	.81±.09
aaBB$c^{ch}c^{ch}$ddPP	47±6	73±5	.64±.10
aaBBCCDDpp	32±2	71±5	.45±.04
aaBBCCddpp	9±3	59±4	.17±.05
aaBB$c^{ch}c^{ch}$DDpp	1±1	55±4	.02±.02
aaBB$c^{e}c^{e}$DDPP	19±2	42±3	.45±.06
aabbCCDDPP	82±3	95±4	.86±.05
aabb$c^{ch}c^{ch}$DDPP	79±3	88±3	.90±.05
aabbCCDDpp	30±2	60±4	.50±.05
aabbCCddPP	40±4	53±4	.75±.10
aabbCCddpp	7±3	53±5	.13±.06
aabb$c^{e}c^{e}$DDPP	20±2	52±5	.38±.05
A^{y}aBBCCDDPP	47±2	47±2	1.00±.06
A^{y}aBBCCDDpp	41±2	45±3	.91±.08
A^{y}abbCCDDpp	44±2	44±2	1.00±.07
A^{y}aBBCCddpp	31±4	44±3	.70±.10
A^{y}abbCCDDPP	43±3	43±3	1.00±.10
A^{y}abbCCddpp	29±2	42±3	.70±.06
A^{y}aBBCCddPP	33±4	41±4	.80±.13
A^{y}abbCCddPP	29±2	33±3	.90±.10
A^{y}aBB$c^{ch}c^{ch}$DDPP	22±2	30±1	.73±.07
A^{y}abb$c^{ch}c^{ch}$DDPP	18±1	24±2	.75±.08
A^{y}aBB$c^{ch}c^{ch}$DDpp	13±2	23±4	.57±.13
A^{y}aBB$c^{ch}c^{ch}$ddPP	11±3	16±3	.70±.23

fewer pigment granules per medullary cell at the tip than at the highest point in the hair, anywhere from the 20th to the 50th field. (The ratio of 10th field count to highest count differs significantly from 1.00.) It is clear from table 2 and figure 2, where, within each color series the genotypes are arranged in descending order of highest medullary number, that pigmentation lag is more conspicuous as the total amount of pigment formed at the highest point drops. In the most heavily pigmented types in both non-agoutis and yellows the value for the 10th field is practically as high as are those found further down the hair axis. As the highest value attained by a given genotype decreases, the value at the tip of the hair decreases still more, and the extent by which the drop in

tip value exceeds the drop in highest value attained is a measure of the amount of pigmentation lag. In some cases it reaches dramatic proportions. For example, the value for the 10th field of *aaBBc^{ch}c^{ch}DDpp* is 1 ± 1, while that for the highest (40th) field is 55 ± 4.

The easiest way to study the general effects of degree of pigmentation is to examine regions of single hairs differing significantly in medullary number, observing the other granule attributes in each region. As this change in medullary number along the axis can be observed only in whole mounts, some estimation of the attributes of individual granules must replace the careful measurements possible in cross-sections (that is, greater diameter and color grade determinations cannot be as accurate where granules overlap). In the 17 genotypes with significant pigmentation lag, changes in the following attributes were found to accompany changes in the number of granules per medullary cell:

1. *Granule size.* In three non-agouti black genotypes (*aaBBc^{e}c^{e}DDPP*, *aaBBCCddPP*, and *aaBBc^{ch}c^{ch}ddPP*) with regularly shaped granules, there was an obvious increase in granule size from the tip to the base of the hair. (It should be noted that the mean granule size of regularly shaped black-fuscous granules in the four genotypes tested ranges from 0.94 μ to 1.44 μ.) In the four non-agouti brown genotypes with regular-shaped granules, however, there was no conspicuous change in granule size along the hair axis (the range of mean sizes for each genotype of round brown granules is narrow, from 0.77 μ to 0.98 μ). In three other black-fuscous types and two other browns, all with shred-shaped granules, no basal increase in size was found (the total range in mean granule size among all shred-shaped types is only from 0.61 μ to 0.73 μ.

The first granules to appear in the most noticeably lagging yellow genotype (*A^{v}aBBc^{ch}c^{ch}DDpp*) are very small, and there is an obvious increase in size accompanying the increase in number (chinchilla yellow granules are always significantly smaller than those of full-color yellows). As the juxtaposition of the situation within single hairs and among genotypes (in parentheses) clearly demonstrates, in situations where any considerable difference in granule size has been found *among* genotypes, a change has also been found *within* single genotypes with a change in medullary number.

2. *Color Intensity.* In the three black-fuscous types with changing granule size, the color intensity increases basally, along with size and number. No change in intensity was found, however, in the shred-granuled genotypes, and no definite change was noted in the yellow or brown genotypes. This fits closely with the relationship between granule size and color intensity determined from comparisons among genotypes.

3. *Granule Shape.* In *aaBBCCddPP*, *aaBBc^{ch}c^{ch}ddPP*, and *aaBBc^{e}c^{e}DDPP*, the larger granules in the more crowded regions are generally more elongated than the smaller ones near the tip, corroborating the observation from comparisons among black-fuscous genotypes of relation between granule shape and size.

4. *Distal arrangement.* The correlation of distal arrangement and number of

granules per medullary cell is complete. In the regions with 60 to 70 granules per medullary cell, there is a tiny proximal clear space; in regions with 40–50 granules per medullary cell the proximal space is larger; and in those with 20–30 granules the proximal space fills most of the cell (the types with no tendency to distal arrangement never have pigmentation lag).

5. *Cortical number.* It is impossible to study variations of cortical number with changes in pigment level, since the cortical pigment is concentrated at the tip of the hair, but as might be expected, the number of granules found in a unit volume of cortex is more closely related to the number of granules per medullary cell produced near the tip of the hair than to that near the base. The highest cortical counts are found in the genotypes with the highest tip medullary number in all series. The most intense browns, which have fewer medullary granules at the tip than do the highest blacks, also have significantly fewer granules per unit volume of cortex at the fourth field. There are three eumelanotic genotypes with no cortical pigment, and they are also the three with the lowest 10th field medullary count (1+1, 7+3, and 9+3).

From this analysis of pigmentation lag and accompanying changes in granule attributes, the concept emerges of a group of characteristics (medullary number, granule size, granule shape, color intensity, distal arrangement, and probably cortical number), all mutually interdependent and varying with the degree of the pigmentation reaction.

CLUMPING

The interrelationships of all variable granule attributes except clumping have now been discussed. As mentioned in the introduction, clumping in mouse hair is of two types. As the first, or flocculent, type of clumping is found wherever there are shred-shaped granules and nowhere else, it is safe to conclude that this type of clumping is either a consequence of the shred-shape or that the two phenomena have a common cause. Granular clumping, however, has no such clear relation to any other attribute. Granular clumps, which appear to be aggregates of fused individual granules, are found in the dilute (*dd*) genotypes of all three color series, with all grades of intensity except intense black, with a wide range of mean granule sizes (0.67 μ up to 1.23 μ greater diameter), and with all granule shapes except long oval. They are very large bodies, with mean volume values ranging in four dilute genotypes from 40.5 μ^3 to 58.2 μ^3, in contrast to individual granule volume values in the same types of 0.50 to 1.61 μ^3 (E. S. RUSSELL 1948) and they occur with approximately the same frequency in all ten dilute types tested (mean, 1.1 percent of pigment bodies). In dilutes, one third to two thirds of the pigment is probably deposited in these clumps. Although granular clumping appears to be an attribute remarkably independent of the others studied here, it is usually accompanied by certain other characteristics. The non-clumped granules of dilutes are somewhat irregularly arranged, with unusual variations in numbers of granules per cell; there is almost always a very low number of cortical granules in proportion to medullary number; there is always some degree of pig-

mentation lag; and there appears to be a certain damping of the upper limit of medullary number. These may quite possibly all be due in some way to the concentration of large proportions of the pigment in the clumps.

Thus we have found the two types of clumping of mouse hair pigment at the opposite poles of dependency relationship. Flocculent clumping is completely dependent upon the presence of shred-shaped granules. Granular clumping, however, appears to be almost completely independent of other granule attributes, including color, and the factors determining it probably act at a different level of organization from those affecting the other independent attributes.

DISCUSSION

In the first paper of this series concerning the quantitative histological analysis of the pigments found in the coat of the house mouse (E. S. RUSSELL 1946), seven main variable granule attributes, together with two subordinate characteristics, all contributing to the visible color differences among the genotypes were described. The final purpose of this work is to find as much as possible about the basic nature of the actions of five different allelic series of genes (agouti series, albino series, black/brown, intense/pink-eye dilution, intense/straight dilution) from the variations with different genotypic combinations of these seven attributes of the pigment granules which are the final products of the action of the coat-color genes.

In many instances, single genic substitutions alter a number of different granule attributes. Also, different granule attributes are affected when the same genic substitution is made against different genotypic backgrounds. Are all these effects independent of each other? If so, the genes concerned have a number of independent actions, and further analysis will be very difficult. If, however, some basic consistency can be found among the multiple effects of a single genic substitution, a closer approach will have been made to an understanding of the basic nature of the action of the alleles concerned. Perhaps limitations upon a certain granule characteristic are set so firmly by the action of one gene that another can have no effect on that particular attribute in the presence of the first, although it would otherwise do so. Or perhaps the conditions set by the presence of one gene simply limit the extent to which other genes can alter granule characteristics. Or perhaps some of these characteristics described here are completely dependent in their variation upon another attribute and have no real independent entity. Still another possibility is that several attributes, although not completely interdependent, have a tendency to vary together in response to changes in some outside factor, either genic or environmental. It is hoped that the attempt made to find all of the interdependences among the variable granule attributes will show some key characteristics, varying relatively independently of each other, and controlling the variations of lesser attributes. If the attempt is successful, it should be possible in a fourth paper to relate the basic action of each of these five allelic series to change in these key pigment attributes.

It is clear from the presentation in this paper that the nature of granule color is one of the key pigmentation characteristics. The color type of the granules being formed at a given locus seems to be determined by no other granule attribute, but rather to determine limitations upon variations in medullary and cortical number, granule size, shape and color intensity. These dependent attributes vary little within the first, or yellow, series, much more within the second, or black-fuscous, series, and to an intermediate degree in the third, or brown, series. There is evidence indicating probable chemical differences between the yellow and non-agouti pigments (stability of yellow versus lability of black-fuscous) and also evidence of greater efficiency of pigment production in the eumelanotic types (highest pigment volume value 1332 in black types, 281 in yellows). It is possible that the lower amount of pigment found in xanthic types may result simply from the requirement of a larger amount of certain components (pigment-radical, specific protein or amino acid) for the same amount of end-product, that is, a lesser efficiency of pigment production.

The situation is not so simple in eumelanotic types, since a distinction must be made between limitations upon granule number and upon the total mass of pigment formed. There does seem to be an upper limit to the sites of pigment deposition within a medullary cell, since the highest number of medullary granules formed in the four highest eumelanotic types (two black-fuscous, two browns) show no significant differences (table 2). LÜBNOW (1939) and DANNEEL (1941) believe that the mitochondria are sites of pigment deposition; possibly all of them present in a single cell are active at both the chinchilla and full-color levels of eumelanotic pigmentation, so that greater deposition can be achieved only by adding more pigment to an already-formed granule. In these four cases, however, although the number is the same the mass of material formed is not. The mean greater diameters of all round brown granules are very similar, and the total pigment volume values in the two particular brown genotypes involved here are close (full-color brown, 480 μ^3, chinchilla brown, 431 μ^3). This is not true in the two black-fuscous types (full-color black, 1332μ^3, chinchilla, 705μ^3). In this case it is clear that a larger mass of material has been formed in the black-fuscous types than in the browns, and by far the largest mass in the full color black. We have two possible and not necessarily mutually exclusive explanations for the difference between the two series. It may be that the brown pigment is chemically different from the black-fuscous pigment, and a difference in efficiency between the brown and black processes may result in exhaustion of all material available for brown pigment at the chinchilla level. Or it may be that chemical or physical differences between the black-fuscous and brown pigments may limit the size which brown granules can reach, so that although excess of all pigment components may be present in the matrix cell, once all sites of pigment deposition are occupied by full-sized brown granules, no more pigment can be formed.

An attempt has been made to analyze the nature of the dependence of granule number and size upon the first of the key pigmentation characteristics, and it is suggested that the upper limits of number may be set in the

black-fuscous series by a limitation in sites of pigment deposition; in browns, by exhaustion of material and/or limitation of sites of deposition; and in yellows by exhaustion of material. The differences in size and shape possible in the three series probably result from chemical differences.

A second relatively independent attribute is granule size. This is limited, as noted above, by color series, but aside from this limitation, the variation in granule size is not conditioned by other granule attributes, but rather controls granule shape and color intensity. The relationship between size and shape has been shown to be practically independent of color series, the largest granules always being oval, the next round, and the smallest shred-shaped. Nongenetic changes in granule size with pigmentation lag also lead to parallel changes in granule shape and color intensity. Flocculent clumping, which has been shown to be invariably associated with shred-shaped granules, is thus secondarily dependent upon changes in granule size.

The third key pigmentation characteristic can not be described as a single granule attribute, but rather as a group phenomenon in which a number of attributes vary together in response to one basic factor, the general degree of pigmentation, whose best characterization between genotypes is the highest mean medullary number achieved at any level in the hair, and within a single genotype the mean medullary number at any particular level. The attributes conditioned by this key factor are medullary number itself, degree of pigmentation lag, cortical number, tendency to distal arrangement, and size of black-fuscous granules. These attributes vary with changes in the general degree of pigmentation, whether such changes are produced by genic substitution or by nongenetic effects such as position along the hair axis.

The final key pigmentation characteristic is the tendency to granular clumping, which appears to be completely independent of the other key characters and to have very slight or irregular influence on the variations of secondary attributes. The few limitations on the variation of other attributes usually associated with granular clumping probably result from the concentration of large proportions of the pigment in enormous clumps.

Thus there are four major relatively independent pigmentation characteristics: nature of color, granule size, degree of pigmentation, and tendency to granular clumping. The action of five allelic series of genes is being studied (agouti series, albino series, black/brown, intense/dilute, and intense/pink-eye dilute). In the fourth paper of this series an attempt will be made to relate key pigmentation characteristics to the action of particular series of alleles.

SUMMARY

1. The three color series into which the granules of mouse hair may be classified (yellows, black-fuscous, and brown) determine upper limits of medullary and cortical number, and limit the extent of variation in granule size, shape, and color intensity.

2. The size of pigment granules controls their shape and their color intensity. To a large extent this is independent of the nature of the color. Granule size,

and consequently shape and color intensity, vary much more in black-fuscous types than in browns, and more in browns than in yellows.

3. Wherever the degree of pigmentation is altered either by genic substitution or by pigmentation lag (hesitation in commencement of pigmentation), several granule attributes vary simultaneously: a. Medullary granule number. b. Granule size in all regular-shaped black-fuscous types and in many yellows. c. Color intensity in regular-shaped black-fuscous types. d. Granule shape in regular-shaped black-fuscous types. e. Tendency to distal arrangement. f. Cortical number.

4. Granular clumping is independent of other granule attributes, but its presence usually alters a few attributes slightly, probably simply by concentrating large proportions of the pigment in large clumps.

5. There appear to be four key pigmentation characteristics, relatively independent of each other and determining the variations of subordinate attributes: a. Nature of granule color. b. Granule size. c. Degree of pigmentation. d. Granular clumping.

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A QUANTITATIVE HISTOLOGICAL STUDY OF THE PIGMENT FOUND IN THE COAT-COLOR MUTANTS OF THE HOUSE MOUSE. IV. THE NATURE OF THE EFFECTS OF GENIC SUBSTITUTION IN FIVE MAJOR ALLELIC SERIES[1]

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THE physiological genetics of the coat-color of mammals has been studied extensively (WRIGHT 1941a, summary; WRIGHT 1941b, 1942; W. L. RUSSELL, E. S. RUSSELL, and L. R. BRAUCH 1947) because local determinations of characteristics and continuation of gene action throughout life provide easily available material for analysis of what must be a relatively short chain of reactions between original gene action and final character expression. This analysis may be based either on description: chemical (DUNN and EINSELE 1938; E. S. RUSSELL 1939; SERRA 1946, 1947); spectrophotometric (DANIEL 1938; BAKER and ANDREWS 1944; SERRA 1947); colorimetric (HEIDENTHAL 1940; E. S. RUSSELL 1939; BRAUCH and RUSSELL 1946b) or histological (WERNEKE 1916; LÜHRING 1928; HUNT and WRIGHT 1918; DANNEEL 1936; SCHILLING 1936; KALISS 1942) of the end-product, or on physiological or chemical studies of intermediate reactions (DANNEEL and SCHAUMANN 1938; W. L. RUSSELL 1939; GINSBURG 1944; L. B. RUSSELL and W. L. RUSSELL 1948). A thorough study of the histology of the pigment granules deposited in various mutants will provide a framework into which deductions from other studies must fit if they represent a true picture of some stage in the pigmentation reactions. Although the conclusions from histological analysis are necessarily limited, they are nevertheless valuable for two reasons. First, the conclusions can be very definite, since the pigment studied is in normal position and completely unaltered by the slide preparation (E. S. RUSSELL 1946). Also, histological analysis can apparently be more detailed and more exactly quantitative than analysis by other methods at present available. In the first paper of this series (E. S. RUSSELL 1946) a picture has been presented of the pigment granules found in 36 different mutant coat-color types in the mouse, and seven attributes varying among the genotypes have been described (number per medullary cell at intervals along the hair axis, number per unit cortical volume at the same intervals, tendency to distal arrangement of granules within medullary cells, granule size, shape, tendency to clumping of either flocculent or granular type, color and intensity of color). Data on certain of these attributes were combined in a second paper (E. S. RUSSELL 1948)

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to give the best estimate possible from histological study of the total volume of pigment in each genotype. For definition of terms and complete measurements the reader is referred to these two papers. In the third paper (E. S. RUSSELL 1949) we have studied the interrelationships among the variations of the seven granule attributes and have concluded that there are four key pigmentation characteristics, varying relatively independently of each other. These are nature of granule color, which imposes limits on granule number, size, and shape; granule size, which determines shape and color intensity; granular clumping, which has very little relation to other attributes; and level of pigmentation, the key factor determining medullary number, degree of pigmentation lag (fewer granules near tip of hair), cortical number, tendency to distal arrangement, and granule size in the black-fuscous series.

The purpose of this paper is to determine from the locations of the observed variations of these four key pigment characteristics among the genotypes, the relation between them and the basic action of the five allelic series of genes involved in these studies, which are the agouti series (A^y, A^w, and a have been tested), the albino series (C, c^{ch}, c^e, and c^a), black-brown (B and b), dark-eye/pink-eye dilution (P and p), and intense-straight dilution (D and d). Throughout the work the possible effect of minor modifying effects has been kept in mind, although no attempt will be made to analyze it in detail.

THE AGOUTI SERIES (A^y, A^w, a)

The genes of the agouti series determine the nature of the pigment produced at any particular level of the hair. In the presence of the dominant A^y gene practically all of the pigment through the whole length of the hair is xanthin, the only exception being a small amount of eumelanotic cortical pigment in sooty yellows. All of the pigment in recessive *aa* genotypes is eumelanin. The apical pigment, both cortical and medullary, of the secondary dominant A^w type is eumelanin, and this is replaced at the 6th to 8th microscopic field by a band of xanthin which persists until the 15th to 18th field, reverting thereafter to eumelanin. In bicolored types, both agoutis and sooty yellows, the attributes of the granules formed at any point along the hair depend on (1) quality (color) of the pigment, (2) the effects in the particular region involved of changes in pigmentation level along the hair axis, and (3) specific effects of other pigment genes. In the eumelanotic tip and basal regions of agouti genotypes the pigment granules are identical in appearance and number with those in the same regions of the corresponding non-agouti genotypes. Similarly, in the xanthic subterminal band there is exact agreement with the situation in the 6th to 18th microscopic fields of corresponding yellow genotypes. This shift was observed carefully in six different agouti genotypes, including three where the apical portions of both the *aa* and A^ya counterparts are pigmented ($A^wA^wBBCCDDPP$, $A^wA^wbbCCDDPP$, *and* $A^wA^wBBCCDDpp$), two where the apex of the *aa* type is pigmented, that of the A^ya pigment free ($A^wA^wBBc^ec^eDDPP$ and $A^wA^wbbc^ec^eDDPP$), and one where the apex of the *aa* type is pigment-free while that of the A^ya counterpart contains pigment ($A^wA^wBBc^{ch}c^{ch}DDpp$).

TABLE 1

Comparison of the pigment in agouti genotypes with that in corresponding yellow and non-agouti types. The numbers are the mean numbers of granules per unit volume at the level listed.

GENETIC BACKGROUND	AGOUTI SERIES	APICAL EUMELANIN	SUBTERMINAL XANTHIN			BASAL EUMELANIN			
		CORT. 4TH	CORT. 10TH	MED. 10TH	COLOR	CORT. 20TH	MED. 20TH	COLOR	SHAPE
BBCCDDPP	A^va	39	12	47	aniline yellow	3	47	aniline yellow	round
	A^vA^v	201	11	43	aniline yellow	26	99	full black	long oval
	aa	219	113	93	full black	21	95	full black	long oval
BBc^{c}c^{c}DDPP	A^va	5	0	0	—	0	0	—	—
	A^vA^v	50	0	3	—	0	34	fuscous	round
	aa	26	2	19	fuscous	0	38	fuscous	round
bbCCDDPP	A^va	35	19	43	aniline yellow	2	43	aniline yellow	round
	A^vA^v	150	12	45	aniline yellow	18	85	carob brown	round
	aa	160	73	82	carob brown	21	90	carob brown	round
bbc^{c}c^{c}DDPP	A^va	0	0	0	—	0	0	—	—
	A^vA^v	38	0	2	—	0	32	carob brown	round
	aa	31	2	20	carob brown	1	34	carob brown	round
BBCCDDpp	A^va	14	5	41	aniline yellow	0	45	aniline yellow	round
	A^vA^v	49	8	52	aniline yellow	3	65	fuscous	shred
	aa	18	15	32	fuscous	4	51	fuscous	shred
BBc^{ch}c^{ch}DDpp	A^va	0	0	13	aniline yellow	0	23	aniline yellow	round
	A^vA^v	0	0	19	aniline yellow	0	32	fuscous	shred
	aa	0	0	1	fuscous	0	19	fuscous	shred

The tabulation of these data (table 1) makes obvious the correspondence of number, color, and shape of granules, but does not establish the relationships of granule size, which can not be measured accurately at each level in the hair, but only in cross-sections coming from anywhere between the 15th and 45th microscopic field of the individual hairs. Such sections were made of two of the agouti genotypes, *A^vA^vBBCCDDPP* and *A^vA^vBBc^{ch}c^{ch}DDpp*. The mean

greater diameter of the yellow granules in the full-color agouti was .85 μ, that in the corresponding full-color yellow, .83 μ, and the mean greater diameter of the black granules was 1.38 μ, as against 1.44 μ in full-color blacks. The mean greater diameter of yellow granules in pink-eyed chinchilla agoutis was .73 μ, while the diameter in pink-eyed chinchilla yellow was 0.66 μ; the mean greater diameter of eumelanotic granules in this type was 0.57 μ, and in the corresponding non-agouti, .62 μ. Thus in these two tested genotypes, the size of the granules as well as their other attributes have been shown to fit closely with those of the same type of granules in corresponding non-agoutis and yellows.

The rapid shift from eumelanotic to xanthic pigment deposition in the space of two or three medullary cells has been described by KALISS (1942). Frequently both types of granules appear in the same medullary cell at the point of transition.

In a previous paper (E. S. RUSSELL 1949) the very fundamental nature of the difference between eumelanotic and xanthic pigmentation was stressed. Yet this study of the agouti series genes and the banding of A^w-genotypes shows that basic as these differences are, some trigger mechanism can shift the pigmentation reaction completely from one to the other within the space of two or three medullary cells. The action of the agouti series genes must be to control this trigger.

THE ALBINO SERIES

The genes of the albino series change the degree of pigmentation. That is, they change the total amount of pigment without changing its type. In this way their action resembles the general change in level of pigmentation described in the third paper of this series as one of the key pigmentation characteristics. Both processes affect a wide variety of granule attributes, and their effects vary with the background. If the nature of the change of these attributes with albino series substitutions is the same as that with proven cases of non-genic changes in level, we may conclude that the action of the albino series is a general one, affecting the level of the whole pigmentation reaction.

To test this possibility table 2 compares the effects of changes in pigmentation level with those of substituting homozygous chinchilla and extreme dilute for full-color on four different types of genetic background. The value for the genic substitution is expressed as the mean of the ratio of the value with the lower alleles to that with full color. The values used for the medullary and cortical numbers are the sums of the counts at all levels for each genotype. The effect of change in pigmentation level must be expressed in words as accurate measurements could not be made on the whole mounts where this phenomenon had to be observed. In general there is great similarity between the effects of the two types, and where a lowering of the general level of pigmentation reduces the number of granules or decreases their size, albino series substitution does likewise. For example, in dark-eyed blacks, size is reduced by both factors, in pink-eyed blacks by neither.

The excellent general parallelism in effect of these two types of change

TABLE 2

Comparison of the effect upon granule attributes on various genetic backgrounds of changes in pigmentation level and of albino series substitutions. Wherever possible a quantitative valuation is given to the albino-series substitution in the form of a numerical ratio.

GENETIC BACKGROUND	ATTRIBUTE	EFFECT OF CHANGE IN PIGMENTATION LEVEL	EFFECT OF ALBINO-SERIES SUBSTITUTION	
			$c^{ch}c^{ch}/CC$	$c^{e}c^{e}/CC$
all $A^{w}a$ types	medullary number	lowering level causes pigment lag in light types	.39–.62 for different examples	.00
	cortical number	lowering level reduces number	.00–.46 for different examples	.00
	granule size	only extreme low level reduces size	.83–.97 for different examples	no granules
BBPPDD	medullary number, mid-region	lowering level gives strong pigment lag	.99*	.41
	cortical number	lowering level reduces number	1.04*	.06
	granule size	lowering level reduces length	.73	.65
BBppDD	medullary number, mid-region	lowering level gives intensified pigment lag	.63	.00
	cortical number	lowering level gives intensified pigment lag	.00	.00
	granule size	no effect	.97	no granules
bbppDD	medullary number mid-region	lowering level gives strong pigment lag	.97*	.44
	cortical number	lowering level reduces number	.80	.13
	granule size	no effect	1.03	1.00

* These apparently aberrant values are discussed in text.

upon medullary and cortical granule number is marred by two conspicuous apparent exceptions, marked in the table by asterisks. These are substitutions of chinchilla for full-color against two backgrounds which show no significant reduction in granule number. This circumstance has already been studied rather thoroughly in the discussion section of the third paper of this series, where it was postulated that there is an upper limit to the number of sites of pigment deposition per cell. These four genotypes have all reached this possible upper limit, a mean for medullary cells of 85–90 granules. Since pigment-forming activity is already sufficiently intense in the chinchillas to use all available sites, a further increase in activity as might be found in a full-color genotype could not increase granule number. Thus, these apparent exceptions may fit in with the rest. Moreover, the substitution of extreme dilute for full-color on these same backgrounds does show typical reduction in medullary and cortical number, conforming to the general parallelism.

The effects of the two types of change on granule size and accompanying characters (shape, intensity) show complete parallelism. Table 2 gives the data for size only, but where size is reduced on a dark-sepia (*BBPPDD*) background the length of the granules is correspondingly decreased and their intensity diminished. Both level and albino-series substitution alter the size of dark-sepia granules, and neither alters the size of brown granules. The size of yellow granules is affected only at the tip of the most conspicuously lagging genotype, $A^{v}ac^{ch}c^{ch}pp$, and the mean size of yellow granules is slightly decreased by the substitution of chinchilla for full-color.

Thus histological data support the idea that the albino series controls the general level of the whole pigmentation reaction rather than altering its qualitative nature.

THE BROWN GENE

The main effect of substituting *bb* for *BB* is to change the nature of eumelanin from the black, fuscous black, or fuscous of the second color series to the carob-brown or mummy brown of the third color series. This occurs throughout the hair of non-agoutis, in the eumelanotic portions of agoutis, and in the sooty regions of the cortex of yellows. As pigment deposition is more stable in form in the third color series than in the second, there is less variation in granule size and shape in *bb* than in *BB* genotypes (table 3); *bb*/*BB* substitutions may thus sometimes greatly reduce mean greater diameter of granules and in other cases affect this attribute very little (the values for *bb* and *BB* genotypic counterparts are given in table 3, in the form of a ratio, but with the actual values there to stress the stability of brown and the lability of black-fuscous). There is always some significant decrease in granule size with *bb*/*BB* substitution on regular-shaped (*PP*) backgrounds, but the reduction is insignificant on shred-shaped backgrounds (*pp*). The effect on granule shape in the regular-shaped backgrounds naturally corresponds with the effect on size, which determines the elongation of the *BB* counterpart. There is also a slight difference in shape among the irregular shreds, those of non-agouti browns appearing slightly shorter and rounder than those of non-agouti blacks. Color intensity naturally varies less in brown granules than in blacks.

In addition to these changes in eumelanotic regions due to the qualitative alteration of the pigmentation reaction, *bb/BB* substitution may have certain quantitative effects on both non-agoutis and yellows (table 4). On the two less intensely colored yellow grounds ($A^{v}ac^{ch}c^{ch}PPDD$ and $A^{v}aCCPPdd$) the *bb* stocks tested had fewer granules than corresponding *BB* stocks (table 4). A significant effect on cortical granule number was also found on the highest non-agouti backgrounds (*aaCCPPDD* and $aac^{ch}c^{ch}PPDD$), fitting in with a

TABLE 3

The effect of bb/BB substitution upon characteristics of individual granules in non-agouti genotypes.

BACKGROUND	RATIO *bb/BB*		
	SIZE (μ) MEAN S.E.	SHAPE	COLOR
aaCCPPDD	0.77± .007	round	carob-brown
	1.44± .013	long oval	intense black
$aac^{ch}c^{ch}PPDD$	0.79± .008	round	carob-brown
	1.05± .010	oval	fuscous black
$aac^{e}c^{e}PPDD$	0.77± .012	round	carob-brown
	0.94± .010	round	fuscous
aaCCppDD	0.61± .013	short shred	mummy brown
	0.64± .018	long shred	fuscous
aaccPPdd	0.98± .020	round	carob-brown
	1.23± .011	oval	fuscous black
aaCCppdd	0.67± .023	short shred	mummy brown
	0.73± .022	long shred	fuscous

pigmentation lag previously recorded (E. S. RUSSELL 1949, table 2) in these *bb* genotypes and lacking in the corresponding *BB* types. These differences between corresponding *bb* and *BB* stocks may have two explanations. Minor modifiers affecting level of pigmentation may have become fixed in certain stocks but not in others. If this is true, these differences are not related to *bb/BB* substitution as such. However, this substitution itself may cause a very slight lowering of the general level of the pigmentation reaction, producing a significant effect on the final product only where the rest of the genotype produces a level near some critical threshold. If this second alternative is true,

observed effects of the brown alleles could be of two types, but it is still more probable that there is only one basic gene action. The major effect is to determine which of two processes, one leading to pigments of the black-fuscous series, and the other to brown pigments, shall control the eumelanotic pigmentation reaction. If the *bb* phase of such a reaction is slightly less efficient than the *BB* phase, the general level of pigmentation therein could be lower in both yellows and non-agoutis, accounting for this observed minor quantitative effect of *bb/BB* substitution.

TABLE 4

The effect of bb/BB substitutions upon the level of pigmentation in all genotypes tested. Values given are the mean and standard error of the ratio of the value in a bb genotype to that in its BB counterpart.

BACKGROUND	$A^{w}a$ GENOTYPES		*aa* GENOTYPES	
	MED. NO. MID-REGION	CORT. NO. 4TH FIELD	MED. NO. MID-REGION	CORT. NO. 4TH FIELD
CCPPDD	.91± .07	.90± .18	1.00± .03	.73± .04*
$c^{ch}c^{ch}PPDD$	.80± .05*	.92± .30	.99± .03	.72± .04*
CCppDD	.93± .07	1.07± .34	.83± .05*	.94± .23
CCPPdd	.78± .11*	.63± .35	.75± .07*	.84± .12
CCppdd	.98± .07	?	.93± .07	?
$c^{c}c^{c}PPDD$	—	—	1.10± .07	1.55± .31

*= *significant reduction.*

PINK-EYED DILUTION

The effects of *pp/PP* substitution on hair pigments are to decrease the size of the pigment granules of all non-agoutis, changing them to a very characteristic irregular shred shape, with flocculent clumping, and to lower the level of the pigmentation reaction considerably in non-agoutis and slightly in yellows.

The granules in all pink-eyed non-agoutis are very much alike and completely unlike those of any other type. As granule size is one of the key pigmentation characteristics, affecting shape and color intensity, and as the mean greater diameters of granules in *aapp* genotypes are smaller (with one pink-eyed yellow exception) than those of any other genotype (E. S. RUSSELL 1946, 1949 (figure 1)) it is possible that the unique shape of the *aapp* granule is a simple consequence of its small size. However, the great similarity and distinctiveness of the appearance and size distribution of the granules in the five *aapp* genotypes tested suggest that *pp/PP* substitution produces a qualitative alteration of the nature of the eumelanotic pigmentation process. With this possibility in mind, it seems wise to devote space to a more detailed description of *aapp* granules than was given previously (E. S. RUSSELL 1946). WERNEKE (1916) in the only earlier description of *aapp* granules that has been found, says very aptly, "they somewhat resemble the crumbs produced by rubbing paper with India rubber" (translated by GRÜNEBERG, in the *Genetics of the*

Mouse). There are shreds, tiny flecks, and larger flocculent clumps of pigment. The edges of the granules never appear as clear and sharp as in dark-eyed types. The mean greater diameter of all *aapp* genotypes lumped together is $0.65 \pm .01\ \mu$, with means of individual genotypes ranging only from $0.61 \pm .01\ \mu$ to $0.73 \pm .02\ \mu$. The modal granule size (figure 1) is always very small, $0.25-0.50\ \mu$ in one type, 0.50–0.75 μ in the other four. The distributions of granule sizes given for three *aapp* genotypes in figure 1 look very much like normal curves damped at the lower end by the limit of the resolving power of the microscope (0.25 μ). There may be numerous flecks of pigment in pink-

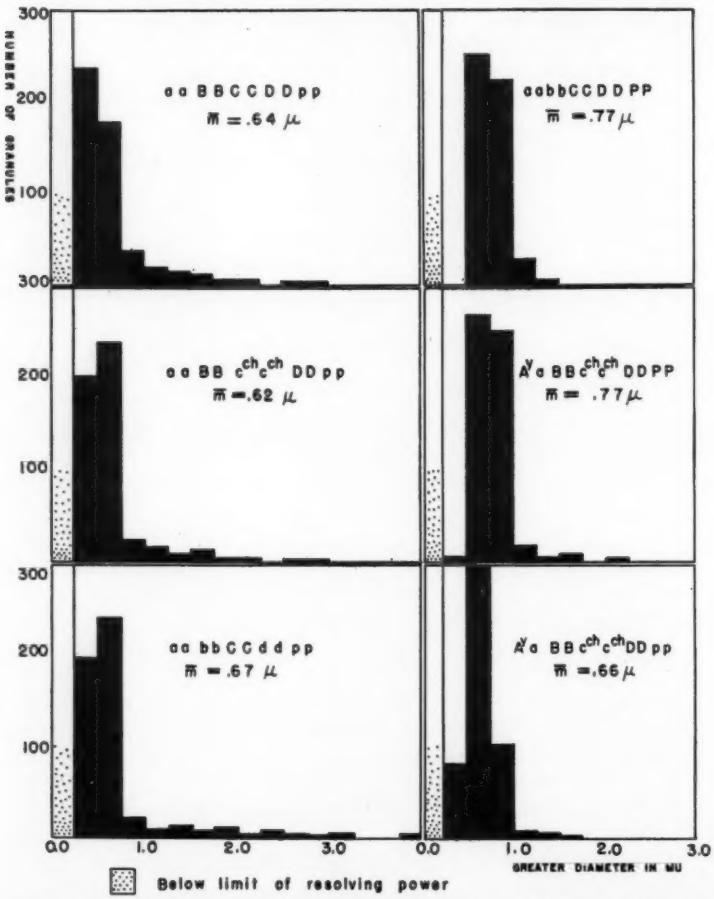

FIG. 1. Chart of greater diameter distribution of 500 granules of six characteristic genotypes, contrasting the type of distribution in non-agouti pink-eyed genotypes (left-hand column) with that in other genotypes with as nearly as possible the same mean greater diameter (right-hand column).

eyed genotypes which are too small to be seen in the conventional microscope. The distributions of granule sizes in the other three genotypes in figure 1 are very different from these, although the mean granule sizes are not very different. In two types (*aabbCCDDPP*, $A^{v}aBBc^{ch}c^{ch}DDpp$) the distribution curve of granule sizes definitely does not overlap the limit of visibility. In the third ($A^{v}aBBc^{ch}c^{ch}DDpp$), the most noticeably lagging yellow genotype, with the lowest mean granule size, there may be a few granules invisible because of their small size, but the overlap is not as great as in *aapp* types. It may be important that this is a pink-eyed yellow. While *aapp* granules are completely unlike all others, their attributes still are affected in a very minor way by certain other genic substitutions: *aabbCCDDpp* granules are on the average shorter and more rounded than those in *aaBBCCDDpp*, showing some relation to the effect of *bb/BB* substitution on a dark-eyed background. Also, in spite of the presence of flocculent clumps in all *aapp* genotypes, granular clumps can be distinguished in *aaBBCCddpp* (figure 1) partly by the mottled appearance of the *dd* intercellular irregularity of distribution but especially by the greatly increased upper limit of size of pigment bodies.

In addition to this effect of *pp/PP* substitution on the manner of eumelanotic pigment deposition, there are several effects on both eumelanotic and xanthic pigmentation which are shown in table 5 to be similar in nature to the general changes in pigmentation level previously described. As a listing of the effects of changes in pigmentation level on various genetic backgrounds has already been given in table 2, in this present table 5 all that is given is the de-

TABLE 5

Comparison of the effect upon granule attributes on various genetic backgrounds of changes in pigmentation level and of pink-eye/dark-eye substitutions. A quantitative value is given to the pp/PP substitution in the form of a numerical ratio.

QUALITY OF COLOR	ATTRIBUTE	EFFECT OF *pp/PP* SUBSTITUTION						COMPARISON TO LEVEL EFFECT
		CCBBDD	*CCbbDD*	$c^{ch}c^{ch}BBDD$	$c^{e}c^{e}BBDD$	*CCBBdd*	*CCbbdd*	
Xanthin	medullary number	.98	.97	.89		1.07	1.26	same
	cortical number	.35	.32	.00		.00	.00	exaggerated in *pp*
	degree of pigment lag	.91	1.00	.78		.88	.77	sometimes exaggerated in *pp*
	granule size	.96± .02	.96± .02	.86± .02		1.03± .02	1.00± .02	same
Eumelanin	medullary number	.67	.58	.43	0.00	.67	.77	same
	cortical number	.10	.11	.00	.00	.00	.00	exaggerated in *pp*
	degree of pigment lag	.46	.58	.02		.21	.19	exaggerated in *pp*
	granule size	.44± .02	.79± .02	.59± .01		.59± .02	.68± .03	specific diet of *pp*

gree of correspondence between the effects of *pp/PP* substitution and level change. As in the albino series comparisons, the values used to determine the ratios of medullary and cortical number between genotypes are the summation of the means at all the levels tested. It should be noted that the effect of *pp/PP* substitution is termed either the same as that of level change, or else there is an exaggerated drop in the *pp* type. This means that with *pp* the drop in number of cortical granules and the increase in degree of pigmentation lag are much greater than would be expected from the drop in total medullary number observed on the same genetic background. These exaggerated effects probably are a specific result of *pp/PP* substitution rather than general quantitative changes in degree of pigmentation, as they do not show the same neat parallelism with level change along the hair which have been described for albino series substitutions.

Thus the pink-eye gene affects the deposition of pigment in all non-agoutis, resulting in a particular characteristic type of very small irregular shreds, flecks, and flocculent clumps. The color quality of the pigment itself is not altered. In addition, *pp/PP* substitution decreases the degree of pigmentation in both non-agoutis and yellows in a distinctive way marked by exaggerated decrease in cortical number and increase in pigmentation lag.

THE DILUTION GENE

The *dd/DD* gene-pair has been misnamed, as the substitution of *dd* for *DD* does not decrease the total volume of pigment (BRAUCH and RUSSELL 1946b, E. S. RUSSELL 1948). Actually there is more pigment in dilutes than in corresponding dark types (table 6). This pigment is, however, distributed unevenly, much of it being concentrated in very large bodies called "granular

TABLE 6

Effects of dd/DD substitution upon amount and distribution of pigment.

GENETIC BACKGROUND	RATIO VOLUME PIGMENT IN MEDULLA, *dd/DD*	PROPORTION TOTAL PIGMENT IN CLUMPS, *dd*	RATIO CORTICAL GRANULE NUMBER, *dd/DD*	RATIO PIGMENT LAG, *dd/DD*
aaBBCCPP	1.06	.28	.27	.83
aaBB$c^{ch}c^{ch}$PP	1.48	.40	.51	.64
aabbCCPP	1.13	.37	.34	.87
aaBBCCpp	(1.65)	(.64)	.05	.38
aabbCCpp	(1.66)	(.78)	.00	.26
A^vaBBCCPP	1.33	.48	.20	.80
A^vaBB$c^{ch}c^{ch}$PP	(1.11)	(.49)	.20	.96
A^vabbCCPP	(1.53)	(.47)	.09	.90
A^vaBBCCpp	(1.76)	(.44)	.00	.77
A^vabbCCpp	(2.56)	(.50)	.00	.70

( ) = value estimated only, since actual measurements of clump volume in these genotypes were lost by fire.

clumps." These clumps have very definite attributes, and are so little influenced by other granule characters that their presence has been termed one of the key pigmentation characteristics (E. S. RUSSELL 1949). They were found in all *dd* genotypes tested, whether black-fuscous, brown, or yellow, with or without pink-eye and its associated flocculent clumping. Although few in number (1.1 percent of all pigmented bodies on the average), these clumps are large and conspicuous, with clear-cut edges which contrast greatly with the flocculence of *pp* clumps, and often show points of more solid color within them as if they were formed by the coherence of many smaller granules. Most of them are located in the medullary cells, but occasionally a few similar large masses are found in the air spaces between the medullary disks. At the base of the hair there are often two or three enormous clumps which cause local enlargement of the hair diameter, and the club-root of *dd* hairs is frequently full of clumped pigment. Although the dilute hairs, like all others, were studied at 1800×, they could be distinguished from all other types at magnifications as low as 200× by these granular clumps and by a general appearance of irregularity in granule arrangement, which has previously been described in the literature (GRÜNEBERG 1943; KEELER 1931). Detailed measurements of 100 granular clumps of each available *dd* genotype were given in the second paper of this series in connection with determinations of total pigment volume. Their average frequency, as mentioned above, was only 1.1 percent of the total number of pigmented bodies observed, but the mean volume values (lw^2/n) are very great, ranging from 40.5 μ^3 to 58.2 μ^3 for different genotypes, in contrast to mean volume-values ranging from 0.50 μ^3 to 1.61 μ^3 in the same genotypes (E. S. RUSSELL 1948, table 2). Actually, between one-third and two-thirds of all pigment in dilute types must be concentrated in these granular clumps (table 6).

As discussed in the paper on key pigmentation characteristics, the few other granule attributes which appear to be associated at all with granular clumping (reduction in cortical number, increase in pigmentation lag, damping of upper limit of medullary number) could all very well be consequences of the same unevenness of pigment distribution which leads to granular clumping. The visually dull diluted effect of all *dd* genotypes can easily be explained by this unevenness. Much less light can be absorbed by a unit volume of pigment concentrated in a giant clump than by the same amount spread out uniformly as 50 small granules. Also, KEELER (1931) in particular has mentioned what must be true, that reduction in cortical pigmentation undoubtedly dulls the coat.

Thus it appears that the basic action of the dilute gene is not to reduce the amount of pigment, but to disarrange it so that large granular clumps and various other uneven distributions appear.

DISCUSSION

In this paper an attempt has been made to determine as much as possible concerning the basic nature of the actions of the genes in five of the main allelic series of mouse coat-color genes: the agouti series, albino series, black/

brown, intense/pink-eyed dilution, and intense/blue dilution. To do this, material from three previous papers in this series has been used, including data on the variations of seven granule attributes recorded for 36 different genotypes in the first paper of the series (E. S. RUSSELL, 1946) estimates of the total volume of pigment in each tested genotype (E. S. RUSSELL 1948) and the analysis in the third paper of the series (E. S. RUSSELL 1949) of the interdependence of these attributes into four groups of variables controlled by four key pigment characteristics, namely, nature of granule color, granule size, granular clumping, and level of pigmentation. If the genes in each of these allelic series have a single basic type of action (WRIGHT 1941, MULLER 1932, GRÜNEBERG 1938), substitutions in each of these series should lead to variations in only one, or at most two, of these key characteristics.

The effect of agouti series substitutions on hair pigment has to do entirely with the first of these key characteristics, nature of granule color. It is to determine whether the pigment produced at a certain level of the hair axis is of the "xanthic" type of the first color series or one of the "eumelanotic" types of the second or third color series. Other characteristics in these regions are, of course, determined by the color series, including limits on granule number, and variations in granule size, shape, and intensity of color. In agouti hairs, the condition may change from the xanthin producing to the eumelanin producing phase within the space of one or two medullary cells, suggesting that the very basic differences between the two reactions are controlled by a trigger mechanism activated by the agouti series genes. In addition to the present histological studies of completed hairs, KALISS's (1942) studies of the growing hair follicle in three to six day old animals are very helpful in this connection. Concerning the yellow band of agouti hairs he writes: "Though the transition from yellow pigment to black pigment takes place within a short distance along the hair shaft, there is no sharp, definitive boundary between the two colors. On the contrary, there is a graded darkening in the granules from septule to septule, so that one can establish a color series from bright yellow to brown yellow, dark-brown yellow, and finally the brown-black melanin that is also found in black hairs. . . . Occasionally one may find both yellow-brown and oval brown-black granules in a single cell or septule." It seems to me that in the full grown hair this gradation is very rapid, and there are few granules which could be classified as intermediate in color.

In addition to his observations on the transition zone of color, KALISS's findings on the comparison of the amount of pigment deposited in yellows and blacks are interesting in relation to the problems of agouti-series action. Although not strictly quantitative, they support the idea, based on more quantitative work, which was developed in the third paper of this series and in the section of this present paper dealing with the agouti series genes. KALISS states, "It is of interest . . . that in general the relative amount of pigment produced by any one cell seems to be less for yellow phenotypes, including the yellow band of the agouti hair, than for blacks, and that one finds less cortical pigment in yellow hairs than in black." The wide range of genotypes in the

present study, especially the inclusion of pink-eyed types, has shown that under certain special circumstances there are exceptions to this generalization. This is most often true where a non-agouti genotype shows marked pigmentation lag, in which case the corresponding yellow genotype shows more pigment near the tip (the subterminal region of $BBc^{ch}c^{ch}DDp\tilde{p}$ types, for example (table 1)). However, in the great majority of cases it is true that there is more pigment in any given region of a non-agouti type than in its yellow counterpart, and that the upper limit of possible amount of pigment deposited is much higher in non-agoutis than in yellows (E. S. RUSSELL 1948).

Knowledge of the actual chemical constitution of mouse xanthin and eumelanin, and of the differences between the two, would do a great deal to help in determining the action of the agouti genes. Analysis of melanins is very difficult, particularly when they must be separated from hair keratins, and so far as I know, no recent analysis has been made of mouse hair melanin. Valuable work has been reported recently by SERRA (1946, 1947) on the constitution of melanin from black and yellow rabbit hair, separated from the keratin by chromatography. He concludes that melanins are "melanoproteids composed of a protein and a melanoid," very closely bound together. The color of melanoids separated from yellow and black melanins is very much the same, but the relative amounts are quite different, 55.5 percent of the black melanin being melanoid, as against 30.3 percent of the yellow. In addition, there is a difference in the proportion of various amino acids in the protein portion. Either type of difference might conceivably account for the differences observed histologically in the upper limit of amounts of pigment, color of pigment granules, and their size and shape. It is very much to be hoped that this type of chemical analysis may be applied to mouse hair melanin.

Whatever factor in melanin formation is altered by agouti series substitution, one which definitely is not is the amount of dopa-oxidase, the oxidative enzyme found, among other places, in the hair follicles of mice, guinea-pigs, and rabbits (W. L. RUSSELL 1939; DANNEEL and SCHAUMANN 1938; L. B. RUSSELL and W. L. RUSSELL 1948). Its concentration has been studied for a wide variety of mouse genotypes by RUSSELL and RUSSELL. They found the concentration varied greatly among the genotypes, but was always the same for corresponding yellow and non-agouti types. In fact, the concentration always paralleled closely the amount of xanthic pigment in the yellow counterpart, a fact which may be of significance to this present discussion.

The effects of albino series substitutions seem to be entirely to change the fourth key pigmentation characteristic, pigmentation level. The effects on all attributes changed by these substitutions (medullary number, cortical number, tendency to distal arrangement, granule size, shape, and color intensity) is the same within each color series as the effect produced there by non-genic changes in the level of pigmentation found within single genotypes in cases of pigmentation lag. The idea that the albino series acts by a quantitative rather than a qualitative change in the pigmentation reaction certainly is not a new one. All of the enzyme work on the mouse (BRAUCH and RUSSELL

1946a; L. B. RUSSELL and W. L. RUSSELL 1948) supports the concept of variations in quantity of dopa-oxidase among the members of the albino series. The chemical extraction work of DUNN and EINSELE (1938) on the mouse showed than in "combination with black, the reduction in intensity of hair color by graded steps from full color (black) to white is accompanied by a parallel grade reduction in the quantity of melanin measured by weight." In addition to extracting and weighing mouse melanin, DUNN and EINSELE (1938) made microscopical examinations of the isolated pigment granules. They noted the difference, already discussed at some length in this present series of papers, between the mean granule size in full color (*CC*) and chinchilla ($c^{ch}c^{ch}$) blacks, also the lack of granule size difference between full color and chinchilla browns. They also noted the similarity in granule number between chinchilla and full color and discovered that observed differences in granule size among the four genotypes studied were sufficient to account for the observed differences in melanin content. Thus they concluded, "The chief tangible factor in this reduction in quantity with albino series substitution is the decrease in size of the pigment granules. Each mutant gene in the *c* series thus exerts a characteristic effect on granule size. It is probable that the type of melanin molecule which is affected by these mutations is the same in all genotypes. As far as our data go, the change brought about by mutations at this locus may be described as alterations in the quantity of melanin produced." These present results contradict their contention that "the chief tangible factor . . . is the decrease in size of pigments granules." Detailed study of a wider range of genotypes, analysis of the potentialities of each of the three color series, and a study of general level changes, have indicated that, while under certain circumstances size of granules may be the only factor which changes with albino series substitutions, under other conditions albino series changes in the level of pigmentation may alter the shape, color intensity, cortical and medullary number, and distal arrangement of the pigment granules.

All the evidence indicates that the albino series genes affect the concentration of dopa-oxidase or some other enzyme so closely allied that it produces a dopa-reaction, and that this concentration determines the intensity of the pigmentation reaction (pigmentation level). Elsewhere in this series of papers it has been shown that very similar quantitative variations in pigmentation level can be produced by non-genic changes, emphasizing the generality of the nature of albino series gene action. It is interesting to speculate on what determines the particular type of changes in granule attributes caused by changes in pigmentation level in each color series. The most obvious changes are those in medullary and cortical granule number, both increasing to the upper limit as pigmentation intensity increases. Accompanying these are changes in pigmentation lag, which increases as pigmentation level decreases. The degree of pigmentation lag and amount of cortical pigment correspond rather closely. It would seem that as more pigmentation activity occurs, as through an increase in C-series enzyme concentration, more centers of pigment deposition become active in each cell and more cells become involved. KALISS, in de-

scribing the first deposition of pigment in the hair of 6-day-old mice (1942), states "The pigmented zone starts just above the border of active mitosis, and . . . the pigment bearing cells are confined to several layers (two to five, as a rule, depending upon the hair type) immediately adjacent to the papilla." It seems that the pigmentation enzyme or some precursor necessary for its formation must originate at the center of the hair follicle (probably in the papilla) and spread out from there. The quantity of this substance present would determine how far it would spread, and therefore how many cell layers would deposit pigment. The number of granules produced within an active cell would depend on the local concentration of enzyme and on the other limitations characterizing that genotype. The correspondence between amount of cortical pigment and degree of pigmentation lag is probably explained by this statement of KALISS; "whether the cortical cells will contain any pigment at all depends upon the number of cell layers that are involved in pigment production, and what the relative allotment of these layers will be to the cortex and medulla of the hair." Apparently the cells forming the tip of the medulla are approximately as far from the center from which the enxyme diffuses as are those destined for the cortex. If the amount of enzyme is very large, as it must be in the case of the completely dominant *C* alleles (WRIGHT 1934; RUSSELL and RUSSELL 1948) the amount reaching the extremities of the follicle is sufficient to allow full pigmentation, but as the amount is decreased, the concentration near the extremities, less than at the center, is insufficient for full pigmentation. In black-fuscous types with regular-shaped granules the size, shape, and color intensity of the pigment granules are also altered by changes in pigmentation level. Presumably increase in enzyme can cause more black-fuscous pigment to be added on to a pre-existing granule in this color series. This phenomenon is not found in any yellows or browns nor in the pink-eyed non-agoutis (with irregular shred-shaped granules); apparently in these types there are definite limitations on size and shape of granules due to factors independent of the albino series. Another attribute affected in all color series by changes in the pigmentation level is the tendency to distal arrangement. A heavily pigmented cell appears to have an even distribution of pigment but with decreasing amounts the pigment becomes restricted to the distal side of the cell, away from the follicle. KALISS (1942) has observed the location of pigment at its first appearance in medullary cells, and reports that the granules are first distributed throughout the cytoplasm, then "as the cells . . . are gradually pushed out of the follicle, their pigment contents aggregate in a mass on the distal side of the nucleus." Presumably in heavily pigmented types it is impossible to crowd all the pigment to the distal side of the most heavily pigmented types.

It has been shown that the effects of brown substitution are of two types, a main effect upon the first key pigmentation characteristic, nature of granule color, and a minor effect upon the fourth key pigmentation characteristic, pigmentation level. The minor effect of *bb/BB* substitution, a slight lowering of the level of pigmentation, produces effects on both non-agouti and yellow

backgrounds which probably lie near critical thresholds where slight changes can give noticeable effects. Although the observed effects of the brown alleles appear to be of two types, it is probable that there is only one basic gene action. The major effect, dependent upon agouti-series gene action in the sense that it is effective only in eumelanotic regions, is to determine which of two processes, one leading to pigments of the black-fuscous series, the other to pigments of the brown series, shall control the pigmentation reaction. The basic nature of this effect may be an alteration of the chemistry of some component of the final pigment, and/or a change in the rate of a process leading to the enlargement and elongation of individual granules, making it effective in *BB* but not in *bb* types. Or it may be that there is less effective substrate available for enlarging granules in *bb* than in *BB* types. Limitation of substrate in browns has been suggested for the guinea-pig by WRIGHT (1942) as the cause of the failure of full-color (*CC*) browns to develop more pigment than $c^{kd}c^{kd}$ browns. However, we have already pointed out that the upper limit of medullary granule number has been reached in the mouse in chinchilla as well as in full color both in blacks and browns. We have suggested that in the presence of *bb* the chemistry of the pigmentation process varies from that in the presence of *BB* in such a way that individual granules can not become enlarged and elongated. Thus, putting these two concepts together, it may be that the failure of increase in pigment between chinchilla and full-color brown may be due to a qualitative chemical limitation of some component of the pigmentation process (due to the action of the *b* gene) combined with the quantitative factor of complete use of all sites of pigment deposition (quite independent of the *b* gene). If this interpretation should prove to be true, brown would certainly not be acting by limiting the substrate.

The major effect of substituting pink-eyed for dark-eyed alleles (*pp*/*PP*) is to alter the manner of deposition of eumelanotic pigments. The granules in all dark-eyed types are relatively large and regular in outline, either ovoid or spherical. In pink-eyed non-agoutis they are irregular shreds, many of them so small as to be near the limit of visibility, a few of them large flocculent clumps (figure 1). It is interesting to speculate on the cause of this irregularity. A great deal of interest has been shown by biochemists recently in the manner of deposition of melanin pigments (H. S. MASON 1947; M. J. KOPAC 1947; HERRMANN and BOSS 1945). The general conclusion seems to be that some sort of a matrix of submicroscopic particles present in the cytoplasm attracts the components necessary for pigmentation to centers where they may interact to form granules. There is also considerable evidence that cytoplasmic granules, including completed melanin granules, may include enzymes (HERMANN and BOSS 1945; G. H. HOGEBOOM, A. CLAUDE, and R. D. HOTCHKISS 1946). It is possible that the submicroscopic particles in *aapp* cells are either absent or modified from those in *aapp* cells in a way that prevents well regulated deposition of eumelanin. This would mean failure to attract to a pigmentation center an amount of some component sufficient for eumelanin, though enough was present in pink-eyed yellow for xanthin formation, as

yellow granules in pink-eyed types are normal in shape. Another possible explanation is that the pink-eye genes are responsible for the formation of some substance essential for the formation of eumelanin, and the amount produced by the *pp* types is either very small or else is delayed in arriving at the site of pigment deposition. It should be emphasized that the postulated deficient material could not be dopa-oxidase, since L. B. RUSSELL and W. L. RUSSELL (1948) have shown conclusively that there is no difference between the dopa-reactions of corresponding non-agouti and yellow genotypes. The suggested deficiency of this substance probably does not alter the composition of the colored portion of the eumelanin molecule, as granule color is not changed. Thus we can postulate that the action of the *pp* genes in producing irregular, shred-shaped granules is either an alteration of the cell matrix or a deficiency in the production of some substance other than dopa-oxidase.

But there is another effect of *pp/PP* substitution which must also be explained. There are fewer pigment granules, or at least fewer *visible* pigment granules, in the medullary cells of all non-agoutis than in their dark-eyed counterparts. Also, in both non-agoutis and yellows, there is considerable reduction in cortical pigment in pink-eyed types and some tendency to pigmentation lag. This lag reaches extreme proportions in some of the lighter non-agouti pink-eyes (ratio of medullary granule number at the 10th field to the full number, *aaBBCCddpp*, .17; *aaBBc^{ch}c^{ch}DDpp*, .02; *aabbCCddpp*, .13). In the discussion of the action of the albino series, the suggestion was made that reduced cortical pigment and pigmentation lag could both result from the failure of an amount of the product of the genes in question sufficient for full pigmentation to diffuse to the outer boundaries of the hair follicle. The same possibility for diffusion (of a different substance) could explain the exaggerated pigmentation lag and cortical reduction of all pink-eyed types. Is it too much to assume that a single gene-product could act quantitatively at the intercellular level, tending to restrict pigment to the inner parts of the follicle, and in addition could act either quantitatively or qualitatively within single cells to alter the manner of pigment deposition? In view of the usually accepted hypothesis of the unitary nature of original gene action (WRIGHT 1941) this is very much to be hoped.

The action of the dilute gene-pair (*Dd*) seems to be concerned entirely with the distribution of pigment granules within and among the cells of the hair. In spite of the dulled diluted appearance of all *dd* types, their total amount of pigment is either as great or greater than that in the corresponding *DD* type (E. S. RUSSELL., 1948; L. R. BRAUCH, and W. L. RUSSELL 1946). Other individual granule attributes such as shape, size, and color are changed little if at all. In all *dd* types the pigment is distributed very unevenly both within and between the cells. The most obvious manifestation of this is the production of very large granular clumps. These are apparently responsible in large part for the visual dilution, as the huge amounts of pigment concentrated in the clumps (one-third to two-thirds of the total, table 6) absorb much less light than they would if spread evenly in the cells. Another manifestation of the gene action

which also helps in dulling the visual color is a reduction in cortical pigment, which is different from that described for pink-eyed and albino-series types in that it is more extreme in proportion to the pigmentation lag in a given genotype. As described earlier in this paper, in addition to the clumps within the medullary cells there are occasional large masses in the air-spaces between the medullary disks, and at the base of the hair there are often two or three enormous clumps which cause local enlargement of the hair diameter, and the club-root may be full of clumped pigment. L. B. RUSSELL and W. L. RUSSELL (1948) have also found that the dopa-reaction in dilute genotypes is "clumped," that is irregular within a single follicle. Two possible explanations of this irregular distribution of pigment granules can be offered. The general irregularity of pigment arrangement, and even apparently of the distribution of at least one enzyme, suggests that some or all precursors of pigment are released to the hair follicle only at intervals and in terrific outbursts. It is of course difficult to postulate what sort of a mechanism could cause this retention and sudden release of pigment components. An alternative explanation is that the physical properties of *dd* cells are such that the submicroscopic particles in the suggested intracellular matrix (KOPAC 1947) have become clumped together. The apparent intercellular nature of *dd* gene action reduces the probability of the second suggestion.

In this discussion an attempt has been made to suggest from these histological observations and from existing literature on melanin formation what the nature of the action of the five major series of genes may be. Where the suggestions reach beyond deductions from actual observations they can be regarded only as ideas thrown out to stimulate further work. What should be taken more seriously are the limitations on the possible nature of the action of these genes derived from actual observations and measurements of the pigment granules. Conclusions contrary to these observations can hardly be tenable. A case in point is the frequent suggestion (BAKER and ANDREWS 1944; NICKERSON 1946) that yellow melanin is simply a more highly oxidized stage of the same reaction chain than is black. The observed differences between xanthic and eumelanotic granules from corresponding genotypes, and the differences in the actions of other genes on the two backgrounds make this suggestion improbable for the mouse. Where references are made to the literature, comparisons of the effects of specific genic substitutions observed here and by other authors have been limited to observations on the house mouse, to eliminate any possible doubt as to gene "homology." In the more general discussion of melanin formation quotations have been made from work on other mammals.

In general the data gathered and the suggestions made concerning modes of gene action are compatible with the idea that each allelic series affects the intensity of a "single primary reaction" (WRIGHT 1941).

SUMMARY

1. The action of the agouti series genes in the mouse (A^{v}, A^{w}, and a) appears to be to control a trigger mechanism which shifts the nature of the color of

pigmentation reversibly, in agoutis within the space of one or two medullary cells, from the eumelanin-producing to the xanthin-producing type of reaction.

2. The action of the albino series (C, c^{ch}, c^{e}, and c^{a}) seems to be a purely quantitative type of change in the level of pigmentation, in no case changing its nature. This suggests that these genes control the amount of some substance necessary for all pigmentation, of whatever color type.

3. The main effect of bb/BB substitution is a qualitative change, found only in eumelanotic regions, from the black, fuscous black, or fuscous of the second color series to the carob brown or mummy brown of the third color series. The minor effect of this substitution, a slight lowering of the level of pigmentation, produces effects on both non-agouti and yellow backgrounds lying near critical thresholds. There is probably no true pleiotropism here, but rather two processes affected by the same original gene action.

4. Two key pigmentation characteristics, size of granules and level of pigmentation, are affected by the action of the Pp alleles. The specific effect pp/PP substitution on granule size, limited to eumelanotic regions, is the production of very small irregular shred-shaped granules and larger flocculent clumps. The effect of this substitution upon level of pigmentation is found in all three color series and differs from the effect of non-genic level change and albino series change in a disproportionately large pigmentation lag. It is probable that these two effects result from a single original gene action.

5. The main effect of dd/DD substitution appears to be the production of an irregularity of pigment deposition resulting in large granular clumps, unevenness of distribution of pigment among the cells, and reduction in cortical pigment.

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THE PRODUCTION OF CHROMOSOME INTERCHANGES IN DROSOPHILA VIRILIS*[1]

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MULLER'S original discovery that ionizing radiations are capable of breaking the chromosome thread was based on investigations carried out with *Drosophila melanogaster*. But in spite of the numerous studies which have been undertaken using this animal, comparatively little progress has been made in the last twenty years in determining the precise manner in which these breaks are formed and the mechanisms involved in the subsequent rejoining of the broken chromosome ends. The cause of this rather slow advance resides in the fact that Drosophila chromosomes can be observed satisfactorily neither during the breakage or joining process nor for a considerable interval of time thereafter. Consequently, any information regarding these processes must be obtained by inference from events taking place at a later time when the ultimate results of the radiation can be accurately determined by genetic analysis or by a cytological study of the salivary gland chromosomes.

The results of the present investigation are derived from experiments in which the sperm of *Drosophila virilis* males are subjected to different dosages of X-radiation. Only a particular type of chromosome rearrangement is studied; namely, exchanges between different chromosomes. The frequency with which these interchanges occur at the different dosage levels provides some insight into the manner in which the breaks are formed. The information obtained has been collected in such a way that both the relative sensitivity of the different chromosomes to breakage and the relative frequency with which certain numbers of chromosomes are involved in the recovered interchanges are revealed. It is possible to gain some insight into the course of events taking place during the joining of the broken chromosome ends by use of these data. Two different groups of experiments of this type have been conducted: in the one group the temperature at the time of irradiation was held at $28° \pm 1°C$, while in the other a temperature of $3° \pm 1°C$ was maintained. The finding of a significant difference in the results of these two groups merely emphasizes the inadequate state of our knowledge concerning the biological action of ionizing radiations on Drosophila chromosomes.

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MATERIAL AND METHODS

Two methods, in general, have been used to determine the rate of induced interchange in Drosophila: the cytological method in which the salivary gland chromosomes of the F_1 larval progeny of irradiated males are examined, and the genetic method which involves an analysis of the chromosome segregation in the F_2 generation. The primary advantages of the cytological method are that, although the original number of breaks cannot be determined, the actual number of these originals breaks which were used in forming the recovered rearrangements can be detected and the exact nature of the rearrangement can be observed. The limitations of this method are centered around the fact that Y chromosome translocations and exchanges between two heterochromatic regions are difficult or impossible to detect. These limitations are quite critical since it has been shown in an extensive study by KAUFMANN (1946a) that, per unit length of salivary gland chromosome, the breaks which form rearrangements are more likely to be located in the proximal heterochromatic region than in the euchromatic regions. By use of the genetic method (used in this study), these limitations can be surmounted at the expense of detailed information regarding the recovered interchanges. The two methods should be considered complementary.

Drosophila virilis was chosen for the present investigation because this species has the simple primitive chromosome configuration of the genus. The metaphase figure as seen in the ganglion cells of the larva consists of five pairs of rods of approximately equal length and a pair of microchromosomes (chromosome 6). This makes possible the study of the interchanges formed between five strictly independent elements of nearly equal length. The wild type stock used in the experiments being reported was derived from the Pasadena *virilis* strain, while the multiple mutant stock containing broken (*b*) on the second chromosome, telescope (*t*) on the third, cardinal (*cd*) on the fourth, and peach (*pe*) on the fifth was synthesized from available mutant strains. A single pair was chosen from each of the parent strains and the offspring of this pair was the stock used throughout the study. The salivary gland chromosomes and the metaphase figures, found in the ganglion cells of the F_1 larvae, from each pair were examined and no chromosomal abnormalities were discernible. A test cross made between these stocks indicated that the chromosomes were segregating at random.

The procedure which follows was used in making the tests. In order to insure that an abundant supply of mature sperm was being treated, the X-radiation was administered to wild type males which were at least eight days old. Immediately after irradiation these males were mated in mass with mature *b*, *t*, *cd*, *pe* females. Three and one-half days after mating, the males were removed and the females were transferred to fresh half-pint culture bottles. Removal of the males was necessary to prevent the use of sperm which had not completed the maturation divisions at the time of irradiation. This time limit is a very conservative one on the basis of the results obtained by DEMEREC and KAUFMANN (1941) with *melanogaster*. Pair matings were made between

the first F_1 males to hatch from the mass cultures and females of the multiple mutant stock. The offspring of the test cross were examined to determine if the segregation of chromosomes Y, 2, 3, 4, and 5 indicates any linkage between these chromosomes. If additional information was needed to confirm the presence of an interchange, the F_2 wild type males were crossed to the mutant stock and a further count of the offspring was obtained. Cytological checks were made in certain cases in which the appearance of aneuploid classes confused the actual chromosome segregation.

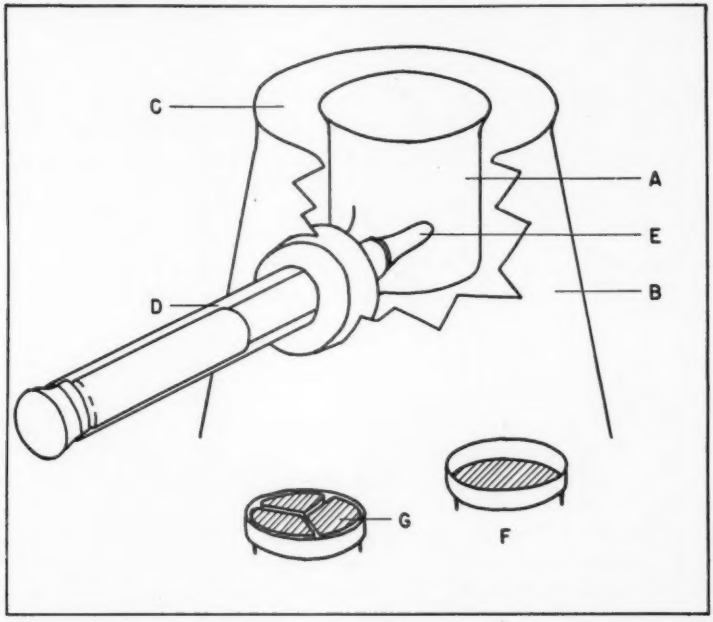

FIGURE 1. A diagram of the irradiation apparatus. A—glass irradiation chamber, B—fluid container, C—temperature controlling fluid, D—glass tube, E—ionization chamber of dosimeter, F—fly container, G—fly capsule.

In the experiments being reported, the flies were maintained at a temperature of either $28° \pm 1°C$ or $3° \pm 1°C$ during irradiation. The apparatus shown in figure 1 was used to insure comparable conditions of incident radiation and dosage measurement in the two sets of experiments. The glass irradiation chamber (A) is inserted into a metal container (B) which contains the temperature controlling fluid (C). The glass tube (D) leading into the irradiation chamber allows the ionization chamber (E) of the dosimeter to be inserted into the position where the flies are treated and also makes possible the insertion of a thermometer into the chamber while the radiation is being administered. Periodic temperature readings were made during treatment of the

flies. In the cold temperature experiments the flies were anaesthetized and placed in a container (F) which was then set on the floor of the irradiation chamber. Although the flies remained anaesthetized during treatment at the cold temperature, it was found necessary to enclose them in a capsule (G) composed of an upper and lower covering of a single thickness of gauze and sides made of light-weight cardboard during irradiation at the warm temperature. Tests show that this layer of gauze does not reduce the radiation to an extent which can be measured with the dosimeter. A fixed 1 mm aluminum filter located directly beneath the Coolidge universal X-ray tube made contact with the top of the irradiation chamber. The flies were kept, therefore, at a constant target distance during the experiment.

The X-radiation was administered to the flies in the following manner. The temperature controlling fluid—a mixture of ice and water or a continuous flow of tap water—was run into the container and maintained at a constant level. After the temperature in the irradiation chamber had become stable, the X-ray machine was calibrated by taking ten readings with a Victoreen condenser type dosimeter whose ionization chamber had been placed in the irradiation chamber. The average of these readings was used to determine the number of roentgen units the machine was delivering per unit time at the point where the flies were to receive the radiation. The flies were then anaesthetized, placed in the chamber, and 1000 r units (based on the initial calibration) were given. After this period some of the flies were removed and mated. Irradiation was continued and a sample of flies was removed and mated after each 1000 r units of treatment until flies receiving 1000, 2000, 3000, and 4000 (cold temperature experiments only) r units had been obtained. Immediately after treatment of the flies, the dosage being delivered by the machine was again checked by ten more readings with the dosimeter. The average of these two sets of readings is used as the calculated dosage administered the flies. The reading given by a condenser type dosimeter varies with temperature introducing, therefore, an instrumental error. As the temperature lowers from 22°C, the meter reads higher than the actual dosage being measured and *vice versa*. Thus in the cold temperature experiments the dosage given is somewhat less than that measured (maximum error is six percent), while in the warm temperature runs the actual dosage is slightly higher.

RESULTS

Before considering the results themselves, it should be recalled that the genetic method of analysis places certain limitations on the type of information which can be obtained. The only rearrangements that are detected are those formed in a sperm in which two or more breaks have occurred in different chromosomes. Furthermore, the resulting interchange of chromosome segments must be such that a viable, fertile zygote is formed. It is obvious that there is no possibility of directly determining the number of breaks produced in a sperm per roentgen unit of radiation. It is possible, however, to determine the minimum number of breaks which were induced in a sperm with a detect-

able interchange since the chromosomes involved in the translocation are known.

Over 800 translocations involving either the Y chromosome or the major autosomes were obtained in this study; therefore, it is not possible to give a complete tabulation of the experimental conditions under which each of these interchanges was produced. However, in table 1 will be found a summary of the number of translocations of different types (in as far as they could be classified by genetic analysis) recovered in all the experiments. The lumping to-

TABLE 1

Number of interchanges recovered of different types.

TYPE OF INTERCHANGE	DOSAGE IN r UNITS					TYPE OF INTERCHANGE	DOSAGE IN r UNITS				
	1000	2000	3000	4000	TOTAL		1000	2000	3000	4000	TOTAL
Y-2	3	8	15	7	33	Y-2-3-4	—	—	1	—	1
Y-3	1	8	21	3	33	Y-2-3-5	—	—	1(2)	1	2(2)
Y-4	4	3	11	7	25	Y-2-4-5	—	—	1	1	2
Y-5	4	10	15	12	41	Y-3-4-5	—	1	1(1)	1	3(1)
2-3	14(2)	20	39	22	95(2)	Y-2 & 3-4	—	—	1	—	1
2-4	8	21	36(1)	16	81(1)	Y-2 & 3-5	—	—(1)	—	—	—(1)
2-5	11	28	45	27	111	Y-2 & 4-5	—	—	1	—	1
3-4	7	28	40(1)	12	87(1)	Y-3 & 2-4	—	—	1	—	1
3-5	11	28	42	18	99	Y-4 & 2-3	—	—(1)	—	—	—(1)
4-5	16	26	29	24	95	Y-4 & 3-5	—	—	1	—	1
Y-2-3	—	1(1)	4(1)	2	7(2)	Y-5 & 2-4	—	2	—	1	3
Y-2-4	—	—(1)	1(2)	—	1(3)	2-3-4-5	—	—	1(1)	1(1)	2(2)
Y-2-5	—	1	2	—	3	2-3 & 4-5	—	1	—	1	2
Y-3-4	—	—	3	—	3	2-4 & 3-5	—	—(1)	1	1	2(1)
Y-4-5	—	1	2	—	3	2-5 & 3-4	—	2	1(1)	1	4(1)
2-3-4	—	3(1)	4(1)	7(1)	14(3)	Y-2-3-4-5	—	—	—(1)	—	—(1)
2-3-5	—	1	8	8	17	Y-2 & 3-4-5	—	—	—	1	1
2-4-5	—	—(1)	5(2)	7(1)	12(4)	Y-5 & 2-3-4	—	—	—(1)	1	1(1)
3-4-5	—	5	10(3)	5(2)	20(5)	Y-4-5 & 2-3	—	—	1	—	1

Note: The figures in parentheses are those cases, not checked cytologically, in which the survival of aneuploid classes confused the chromosome segregation to a limited extent.

gether of the data from both the warm and cold temperature experiments is permissible since the relative frequencies of the different types of translocations induced in the two sets of experiments are essentially the same. The figures given in parentheses are those cases, which were not checked cytologically, in which the survival of aneuploid classes tended to confuse the chromosome segregation to a limited extent. It can be ascertained from the data in this table that chromosome 2 is involved in 398 of the translocations, chromosome 3 in 394, chromosome 4 in 367, chromosome 5 in 423, and the Y chromosome in only 167 of the recovered interchanges. It is to be noted that the inclusion of the figures given in parentheses does not alter appreciably the relative frequency with which these chromosomes are included in an interchange. Consequently, it can be stated as a first approximation that each of the major autosomes is about equally likely to be involved in a given interchange.

The principal information obtained in these experiments is tabulated in table 2. Several things should be noted about the data which are set forth in

TABLE 2

Relation of interchange production to dosage and temperature

EXP. NO.	CHAMBER TEMP. IN °C	DOSAGE IN r UNITS (ca.)	a NUMBER OF SPERM TESTED	b NUMBER OF SPERM WITH INTER-CHANGES	c MINIMUM NO. OF BREAKS IN TESTED SPERM	d b/a	e c/a	f c/b
2	1.7–2.3	1000	140	5	10	0.036	0.071	2.000
		2000	89	10	20	0.112	0.225	2.000
		3000	108	31	65	0.287	0.602	2.097
4	2.3–3.0	982	116	4	8	0.034	0.069	2.000
		1964	115	15	31	0.130	0.270	2.067
		2946	55	17	39	0.309	0.709	2.294
		3928	73	29	64	0.397	0.877	2.207
6	2.0–2.2	1004	129	4	8	0.031	0.062	2.000
		2008	86	8	16	0.093	0.186	2.000
		3012	76	13	31	0.171	0.408	2.385
		4016	95	37	84	0.389	0.884	2.270
7	2.5–2.7	987	195	11	22	0.056	0.113	2.000
		1974	194	33	75	0.170	0.387	2.273
		2961	145	48	105	0.331	0.724	2.188
8	2.0–2.3	1027	184	5	10	0.027	0.054	2.000
		2054	187	26	59	0.139	0.316	2.269
		3081	153	49	110	0.320	0.719	2.245
		4108	145	60	145	0.414	1.000	2.417
9	3.0	997	182	14	28	0.077	0.154	2.000
		1994	182	30	64	0.165	0.352	2.133
		2991	161	52	116	0.323	0.720	2.231
		3988	75	36	82	0.480	1.093	2.278
10	2.0–2.5	987	184	10	20	0.054	0.109	2.000
		1974	168	25	51	0.149	0.304	2.040
		2961	155	48	116	0.310	0.748	2.417
		3948	74	30	66	0.405	0.892	2.200
Comb. cold exps.	1.7–3.0	998	1130	53	106	0.047	0.094	2.000
		1996	1021	147	316	0.144	0.310	2.150
		2986	853	258	582	0.302	0.682	2.256
		4016	462	192	441	0.416	0.955	2.297
11	27.5–28.2	1020	182	10	20	0.055	0.110	2.000
		2040	190	22	47	0.116	0.247	2.136
		3060	150	37	84	0.247	0.560	2.270
12	27.8–28.0	996	185	8	16	0.043	0.086	2.000
		1992	159	22	52	0.138	0.327	2.364
		2988	171	33	70	0.193	0.410	2.121
13	27.9–28.3	1011	183	10	20	0.055	0.109	2.000
		2022	167	14	29	0.084	0.174	2.071
		3033	156	34	77	0.218	0.494	2.265
Comb. warm exps.	27.5–28.3	1009	550	28	56	0.051	0.102	2.000
		2019	516	58	128	0.112	0.248	2.207
		3025	477	104	231	0.218	0.484	2.221

this table. In column c, which is a summation of the minimum number of breaks involved in the observed interchanges, the figures are equal to the sum of the number of chromosomes included in the translocations. Thus if it was found that chromosomes 2, 3, and 5 were linked, it is obvious that at least three breaks must have been induced. Considering column d, it can be seen that upon multiplication of the figures in this column by 100, the percent of the tested sperm containing detectable interchanges is obtained. These data have been plotted in figure 2. The minimum number of detectable breaks per tested sperm is recorded in column e and plotted in figure 3. The average minimum number of breaks in the recovered interchanges is compiled in the

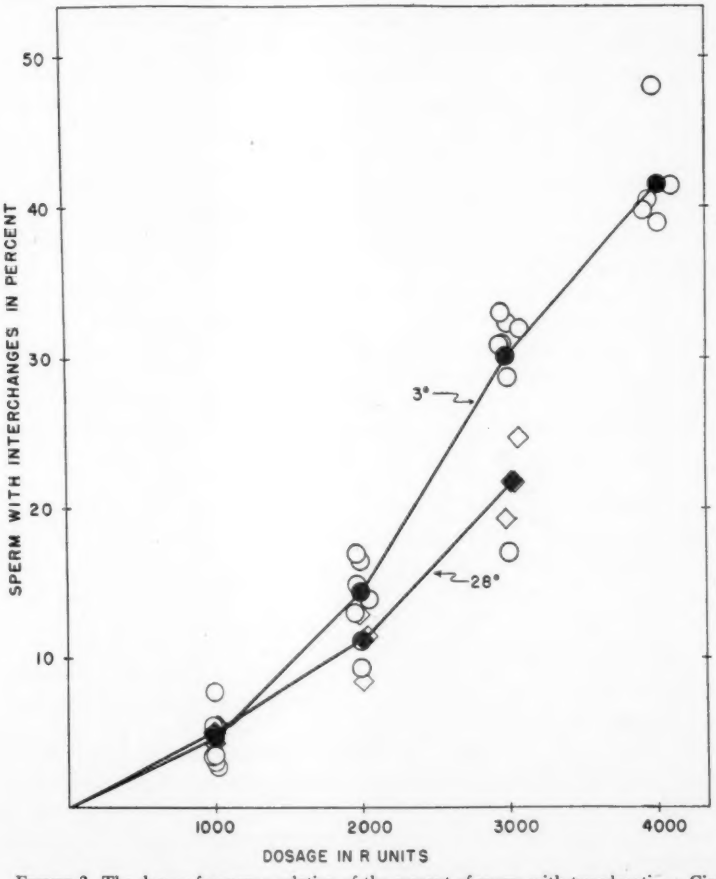

FIGURE 2. The dosage-frequency relation of the percent of sperm with translocations. Circles represent experiments conducted at 3°C, diamonds—28°C. Open symbols represent individual experiments, closed symbols—all experiments combined.

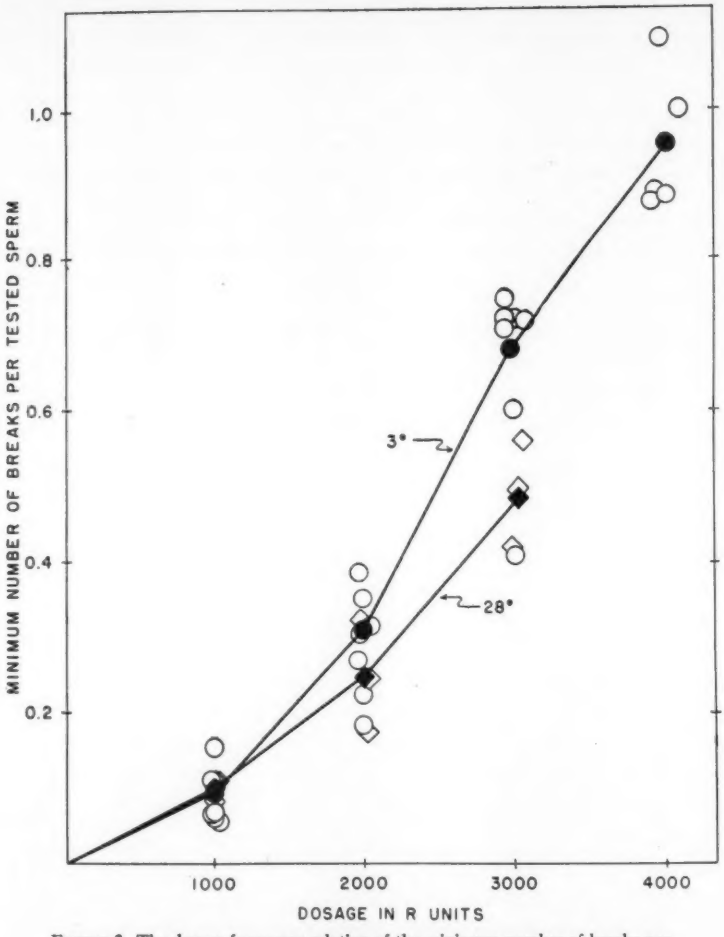

FIGURE 3. The dosage-frequency relation of the minimum number of breaks per tested sperm. See figure 2 for explanation of symbols.

last column of the table. This figure is a measure of the complexity of the rearrangement. The combined results of the individual cold and warm temperature experiments are tabulated below the data given for experiments 10 and 13, respectively. The combined data are represented by solid symbols in figures 2 and 3 while the results of the individual experiments are shown in open symbols.

The data presented in table 2 will be considered first from the standpoint of the relation between dosage and translocation production. From an examination of the sigmoid-shaped curves, for the cold temperature experiments as

presented in figures 2 and 3, it is obvious that over this range of dosage a power function of the form $y = ax^b$ does not adequately fit the empirical results. However, up to 3000 r units in both the warm and cold temperature experiments, the data approximate a power function. By use of the method of least squares, it is found that the exponent of the power curve which best fits the data plotted in figure 2 up to 3000 r units is 1.7 for the experiments performed at 3°C and 1.3 for the 28°C experiments, while for the results represented in figure 3, the exponents are 1.8 and 1.4 respectively. Care should be taken in attributing any broad significance to the particular exponent since it will be apparent from the analysis to follow that it is a function of the temperature and the number of chromosomes being followed as well as a function of dosage and the processes of breakage and union.

The data also show a significant decline in the number of interchanges produced when the temperature of the chamber in which the flies are irradiated is raised 25°C. A chi-square test of independence gives χ^2 values of 2.9 and 11.0 for the pooled data from the experiments in which the treatments were 2000 and 3000 r units respectively. (A chi-square test gives no indication of heterogeneity among the individual experiments at these dosages.) Such values or larger values of chi-square would be expected in less than one out of twelve samples at 2000 r units and less than one out of a thousand samples at 3000 r units if the temperature difference was ineffective. This difference is even more significant than the figures indicate since the dosimeter reads higher than the actual dosage when it is placed in the irradiation chamber held at 3°C. Thus the dosage delivered in the cold experiments is somewhat less than the calculated figure. This temperature effect may be summarized by stating that fewer interchanges are recovered when the sperm are treated at the warm temperature than are recovered when irradiation is carried out at the cold temperature. This effect becomes more pronounced as the dosage delivered to the flies is increased.

It is also evident from the figures presented in the last column of table 2 that, as the dosage is increased, on the average more chromosomes become involved in the interchanges. Therefore, the increase with dosage in the number of breaks per tested sperm is not solely to be attributed to an increase in the number of simple translocations involving only two chromosomes. The data of both the warm and cold temperature experiments indicate that an important factor which contributes to this increase is the parallel rise in the number of chromosomes involved in the interchanges.

ANALYSIS OF RESULTS

It is difficult to evaluate the significance of data such as these unless they can be compared with the results expected on the basis of some plausible theory of chromosome breakage and union. The general argument which follows forms the foundation for determining these expectations. For a given dosage of radiation, there is a probability, p(r), that a particular sperm will contain a total of r chromosome breaks in one or more of the K chromosomes being followed. These r breaks may be distributed among the K chromosomes in

various ways. For example, all r breaks may be in one chromosome or there may be two breaks in one chromosome, (r−2) in another and the remainder of the K chromosomes may not be broken. Therefore let p(d) be the probability that the r breaks will be distributed in a particular way. With each distribution of breaks there is a probability, $p(i_D)$, of detecting (by the genetic method of analysis) the interchanges formed by the union of the broken ends. Now the product $p(d) \cdot p(i_D)$ is the probability of detecting an interchange from a sperm whose r breaks are distributed in one of the possible ways. If this product is summed over all the possible distributions of r breaks among the broken chromosomes, the result is the probability, $S_D{}^r$, of obtaining a detectable interchange from a sperm with r breaks. Since p(r) is the probability that a sperm will have r breaks, then

$$\sum_{r=0}^{\infty} p(r) \cdot S_D{}^r$$

is the probability that for any particular dosage an interchange will be detected. From this figure it is possible to calculate the probability of securing a sperm with a translocation for any dosage if the relation between dosage and breakage is known. The reliability of the theoretical expectations depends on the proper evaluation of p(d), $p(i_D)$, and p(r); therefore, any experimental evidence pertaining to the assumptions made in the evaluation of these probabilities is of primary importance.

In order to determine p(d), it is necessary to know the relative probabilities that each of the chromosomes will be broken. It will be recalled that each of the major autosomes of *virilis* is about equally likely to be included in an interchange. This indicates that each of these chromosomes is equally likely to be broken. Therefore, the probability that there will be r_1 breaks in the second chromosome, r_2 in the third, r_3 in the fourth, and r_4 in the fifth is

$$\frac{r!}{r_1!r_2!r_3!r_4!}\left(\frac{1}{4}\right)^r,$$

where $r_1+r_2+r_3+r_4=r$. Since it is assumed that each of the major autosomes is equally likely to be broken, the above probability is also equal to the probability that there will be r_1 breaks in the third chromosome, r_2 in the fourth, r_3 in the fifth, and r_4 in the second, *etc.* Now if there are t *different* numbers of breaks per chromosome, s_1 of one number, s_2 of another number and s_t of the t^{th} number, then the number of possible arrangements of breaks having equal probabilities is the number of permutations of four things of which s_1 are of one type, s_2 of another type, *etc.* Accordingly, the desired probability that there will be r_1 breaks in one of the autosomes, r_2 in another, r_3 in another, and r_4 in the remaining one is

$$p(d) = \frac{4!}{s_1!s_2!\cdots s_t!}\,\frac{r!}{r_1!r_2!r_3!r_4!}\left(\frac{1}{4}\right)^r.$$

For example, if $r=7$, the probability that there will be three breaks in one chromosome, two breaks each in two other chromosomes, and no breaks in the remaining chromosome (3-2-2-0) is

$$\frac{4!}{1!2!1!}\,\frac{7!}{3!2!2!0!}\left(\frac{1}{4}\right)^7 = 0.15381.$$

It has been shown by BAUER (1942), FANO (1943), and HALDANE and LEA (1947) that if r breaks are formed in k out of the K chromosomes being followed, $2^{r-k}(r-1)!k$ viable arrangements of the broken ends are possible. The term "viable" means that the arrangement is eucentric and euploid. Discounting any induced dominant lethal mutations or any dominant sterility mutations, all of these arrangements should survive in the progeny of the test cross. The next step in the analysis, therefore, is to determine the number of these viable arrangements which are detectable as interchanges using the genetic method of analysis. Obviously, restoration of the original arrangement and any intrachromosomal rearrangements will not be observed. In a chromosome with one break, the only viable intrachromosomal arrangement possible is a restitution of the original arrangement; while in a chromosome with more than one break, the number of such viable arrangements of the broken ends of this chromosome is $2\cdot 4\cdot 6\cdots(2r_1-2)=(r_1-1)!2^{r_1-1}$. Thus the number of detectable interchanges is $2^{r-k}(r-1)!k-2^{r_1-1}(r_1-1)!\cdot 2^{r_2-1}(r_2-1)!\cdots 2^{r_k-1}(r_k-1)!$. If it is assumed that each of the viable arrangements is equally likely to occur, then the probability of obtaining an interchange in the test cross from a sperm with r breaks is obtained by dividing the number of detectable interchanges by the total number of viable arrangements. Upon simplification, this probability becomes

$$p(i_D) = 1 - \frac{(r_1-1)!(r_2-1)!\cdots(r_k-1)!}{k(r-1)!}.$$

The assumption that each of the viable arrangements is equally likely to occur implies not only that a broken end is just as likely to join with a broken end in another chromosome as it is to undergo restitution, but that the union of two or more broken ends in no way affects the joining of other ends except to reduce the number of ends which are available for union. This is a reasonable assumption made purely for the simplification of the mathematics.

In order to make a comparison with the empirical data presented on the minimum number of breaks per tested sperm, it is necessary to determine the expected portion of the detectable interchanges which involve two, three, and four chromosomes. Naturally if $k=0$ or 1, an interchange is impossible, and if $k=2$, all interchanges involve two chromosomes; that is, in the latter case the number of detectable interchanges is equal to the number of two-chromosome interchanges. In the case where $k=3$, the total number of viable interchanges involving only two chromosomes is the sum of the three possible products obtained by multiplying the number of detectable two-chromosome interchanges between any two chromosomes by the number of viable intra-

TABLE 3

Evaluation of theoretical probabilities.

NO. OF BREAKS r	POSSIBLE BREAK DISTRIBUTIONS	PROB. OF SUCH A DISTRIBUTION p(d)	PROB. OF A DETECTABLE INTERCHANGE $p(i_D)$	PROB. OF A 2 CHROMOSOME INTERCHANGE $p(i_2)$	PROB. OF A 3 CHROMOSOME INTERCHANGE $p(i_3)$	PROB. OF A 4 CHROMOSOME INTERCHANGE $p(i_4)$
2	2-0-0-0	.25000	—	—	—	—
	1-1-0-0	.75000	.50000	.50000	—	—
3	3-0-0-0	.11111	—	—	—	—
	1-1-1-0	.22222	.83333	.50000	.33333	—
	2-1-0-0	.66667	.75000	.75000	—	—
4	4-0-0-0	.01563	—	—	—	—
	1-1-1-1	.09375	.95833	.25000	.33333	.37500
	2-2-0-0	.14062	.91667	.91667	—	—
	3-1-0-0	.18750	.83333	.83333	—	—
	2-1-1-0	.56250	.94444	.38889	.55555	—
5	5-0-0-0	.00391	—	—	—	—
	4-1-0-0	.05859	.87500	.87500	—	—
	3-2-0-0	.11719	.95833	.95833	—	—
	3-1-1-0	.23437	.97222	.30555	.66667	—
	2-1-1-1	.23437	.98958	.12500	.33333	.53125
	2-2-1-0	.35156	.98611	.23611	.75000	—
6	6-0-0-0	.00098	—	—	—	—
	5-1-0-0	.01758	.90000	.90000	—	—
	3-3-0-0	.02930	.98333	.98333	—	—
	4-2-0-0	.04395	.97500	.97500	—	—
	4-1-1-0	.08789	.98333	.25000	.73333	—
	2-2-2-0	.08789	.99722	.09167	.90555	—
	3-1-1-1	.11719	.99583	.07500	.30833	.61250
	2-2-1-1	.26367	.99792	.05000	.26667	.68125
	3-2-1-0	.35156	.99444	.17222	.82222	—
7	7-0-0-0	.00024	—	—	—	—
	6-1-0-0	.00513	.91667	.91667	—	—
	5-2-0-0	.01538	.98333	.98333	—	—
	4-3-0-0	.02563	.99167	.99167	—	—
	5-1-1-0	.03076	.98889	.21111	.77778	—
	4-1-1-1	.05127	.99792	.05000	.27917	.66875
	3-3-1-0	.10254	.99815	.12778	.87037	—
	4-2-1-0	.15381	.99722	.13611	.86111	—
	2-2-2-1	.15381	.99965	.01458	.16944	.81563
	3-2-2-0	.15381	.99907	.05278	.94630	—
	3-2-1-1	.30762	.99931	.02778	.22917	.74236
8	8-0-0-0	.00006	—	—	—	—
	7-1-0-0	.00146	0.92857	.92857	—	—
	6-2-0-0	.00513	.98810	.98810	—	—
	4-4-0-0	.00641	.99643	.99643	—	—
	6-1-1-0	.01025	.99206	.18254	.80952	—
	5-3-0-0	.01025	.99524	.99524	—	—
	5-1-1-1	.02051	.99861	.03571	.25238	.71071

TABLE 3 (cont.)

NO. OF BREAKS r	POSSIBLE BREAK DISTRIBUTIONS	PROB. OF SUCH A DISTRIBUTION p(d)	PROB. OF A DETECTABLE INTERCHANGE $p(i_D)$	PROB. OF A 2 CHROMOSOME INTERCHANGE $p(i_2)$	PROB. OF A 3 CHROMOSOME INTERCHANGE $p(i_3)$	PROB. OF A 4 CHROMOSOME INTERCHANGE $p(i_4)$
	2-2-2-2	.03845	.99995	.00327	.06468	.93199
	5-2-1-0	.06152	.99841	.11270	.88571	—
	4-2-2-0	.07690	.99960	.03532	.96429	—
	4-3-1-0	.10254	.99921	.10397	.89524	—
	3-3-2-0	.10254	.99974	.02778	.97196	—
	3-3-1-1	.10254	.99980	.01587	.19603	.78790
	4-2-1-1	.15381	.99970	.01786	.20060	.78125
	3-2-2-1	.30762	.99990	.00675	.13611	.85704
9	9-0-0-0	.00002	—	—	—	—
	8-1-0-0	.00041	.93750	.93750	—	—
	7-2-0-0	.00165	.99107	.99107	—	—
	7-1-1-0	.00329	.99405	.16071	.83333	—
	6-3-0-0	.00385	.99702	.99702	—	—
	5-4-0-0	.00577	.99821	.99821	—	—
	6-1-1-1	.00769	.99926	.02679	.22917	.74330
	6-2-1-0	.02307	.99901	.09623	.90278	—
	3-3-3-0	.02563	.99993	.01171	.98823	—
	4-4-1-0	.02884	.99970	.08720	.91250	—
	5-2-2-0	.03461	.99980	.02560	.97421	—
	5-3-1-0	.04614	.99960	.08849	.91111	—
	5-2-1-1	.06921	.99985	.01250	.17798	.80937
	4-3-2-0	.11536	.99990	.01796	.98194	—
	4-3-1-1	.11536	.99993	.01071	.17292	.81629
	3-2-2-2	.11536	.99999	.00126	.04206	.95666
	4-2-2-1	.17303	.99996	.00379	.11548	.88069
	3-3-2-1	.23071	.99998	.00293	.11012	.88693
10	10-0-0-0	.00000	—	—	—	—
	9-1-0-0	.00011	.94444	.94444	—	—
	8-2-0-0	.00051	.99306	.99306	—	—
	8-1-1-0	.00103	.99537	.14352	.85185	—
	7-3-0-0	.00137	.99802	.99802	—	—
	5-5-0-0	.00144	.99921	.99921	—	—
	6-4-0-0	.00240	.99901	.99901	—	—
	7-1-1-1	.00275	.99950	.02083	.20932	.76934
	7-2-1-0	.00824	.99934	.08399	.91534	—
	6-2-2-0	.01442	.99989	.01951	.98038	—
	6-3-1-0	.01923	.99978	.07738	.92240	—
	6-2-1-1	.02884	.99992	.00901	.15972	.83118
	5-4-1-0	.02884	.99987	.07606	.92381	—
	4-4-2-0	.03605	.99997	.01181	.98816	—
	4-4-1-1	.03605	.99998	.00764	.15427	.83807
	4-3-3-0	.04807	.99998	.00655	.99343	—
	5-3-2-0	.05768	.99996	.01283	.98713	—
	5-3-1-1	.05768	.99997	.00787	.15496	.83714
	3-3-3-1	.06409	.99999	.00106	.09012	.90881
	4-2-2-2	.07210	1.00000	.00062	.03148	.96789
	5-2-2-1	.08652	.99998	.00238	.10053	.89707
	3-3-2-2	.14420	1.00000	.00045	.02589	.97367
	4-3-2-1	.28839	.99999	.00162	.09494	.90343

chromosomal arrangements possible in the third chromosome. The number of detectable interchanges including three chromosomes when $k=3$ is the total number of detectable interchanges minus the number of two-chromosome interchanges. Using similar considerations it is possible to determine the number of interchanges which involve two, three, or four chromosomes for any distribution of breaks among the four chromosomes being followed. Dividing these figures by the number of viable arrangements for the particular distribution of the r breaks, gives the probability of obtaining an interchange involving two chromosomes, $p(i_2)$, three chromosomes, $p(i_3)$, or four chromosomes, $p(i_4)$, where $p(i_D)=p(i_2)+p(i_3)+p(i_4)$. These probabilities along with p(d) for each of the possible break distributions have been calculated and are tabulated in table 3.

The probability, p(r), that for a given dosage a particular sperm will contain r breaks, remains to be evaluated. The Poisson distribution should fulfill the requirements if the breaks are produced individually and collectively at random over the range of dosage used. Since cytological difficulties prevent the observation of single breaks in Drosophila, the substantiating evidence for this assumption must come from other experimental organisms. CARLSON (1941) using the neuroblast cells of Chortophaga, and CATCHESIDE, LEA and THODAY (1946) studying structural changes in Tradescantia, found that the number of single breaks in a cell very closely follows the Poisson distribution. Thus if $\bar{r}$ is the average number of breaks produced by a given dosage, then

$$p(r) = \frac{\bar{r}^r e^{-\bar{r}}}{r!} .$$

This probability involves the assumption of some value for the average number of breaks per unit dosage. In this connection, it is to be noted that in the theoretical considerations so far it has been assumed that all broken ends eventually join. MULLER'S hypothesis (1940) that certain dominant lethal mutations may be caused by single chromosome breaks (the broken ends of the acentric and monocentric fragments fail to undergo union and, on subsequent division of the chromosome, the sister chromatids unite to form an acentric and a dicentric chromosome) has been tested further by PONTECORVO (1941, 1942). In these studies MULLER and PONTECORVO found that at low dosages the frequency of loss of single chromosomes increases linearly with the dosage administered. This provides indirect evidence that some of the dominant lethals are caused by single breaks which fail to unite. If all broken ends have an equal probability of remaining free and this probability is small, then the number of dominant lethals produced by the failure of one or more broken ends to unite should increase almost linearly with moderate dosages as do ordinary gene mutations. Therefore, the figure selected for the "average number of breaks" in the Poisson exponential is $\bar{r}$ multiplied by a coefficient, a, to take into account this particular type of dominant lethal and, in addition, dominant sterility mutations. The $a\bar{r}$ used was chosen such that at 1000 r units the theoretical and empirical values for the percent of sperm with inter-

changes are approximately equal ($a\bar{r} = 0.52$ per 1000 r units of X-radiation). There is some justification for this choice since the constitution of the interchanges induced by 1000 r units is comparatively simple (all of the 81 interchanges produced at this dosage involve only two chromosomes) and thus not subject to the complications invoked by numerous breaks. In addition, this is the point where the results of the warm and the cold temperature experiments nearly coincide.

TABLE 4

The theoretical dosage-frequency relation.

r	S_D^r	S_2^r	S_3^r	S_4^r	1000 r $\bar{ar}=0.52$ p(r)	2000 r $\bar{ar}=1.04$ p(r)	3000 r $\bar{ar}=1.56$ p(r)	4000 r $\bar{ar}=2.08$ p(r)
2	.37500	.37500	—	—	.08038	.19115	.25570	.27025
3	.68518	.61111	.07407	—	.01393	.06627	.13296	.18738
4	.90624	.52734	.34375	.03516	.00181	.01723	.05186	.09743
5	.97005	.34750	.49804	.12451	.00019	.00358	.01618	.04053
6	.99098	.20003	.53954	.25141	.00002	.00062	.00421	.01405
7	.99739	.10724	.50204	.38810	—	.00009	.00094	.00417
8	.99926	.05537	.42889	.51501	—	.00001	.00018	.00109
9	.99979	.02803	.34853	.62328	—	—	.00003	.00025
10	.99999	.01399	.27480	.71117	—	—	—	.00005
				$\sum_{r=2}^{10} S_D^r \cdot p(r) =$	**.04152**	**.13688**	**.25501**	**.37681**
				$\sum_{r=2}^{10} S_2^r \cdot p(r) =$	.03967	.12264	.21106	.28464
				$\sum_{r=2}^{10} S_3^r \cdot p(r) =$	.00175	.01299	.03857	.07780
				$\sum_{r=2}^{10} S_4^r \cdot p(r) =$	.00009	.00126	.00536	.01438
				$2\sum_{r=2}^{10} S_2^r \cdot p(r) + 3\sum_{r=2}^{10} S_3^r \cdot p(r) + 4\sum_{r=2}^{10} S_4^r \cdot p(r) =$	**.08495**	**.28929**	**.55927**	**.86020**

The final assumption to be made is that there is a linear relation between the dosage and the frequency with which breaks are formed. Although this linear relation can be inferred from many types of radiation studies on Drosophila (*e.g.*, BAUER 1942), the direct evidence must rest on the observation of single breaks. The experiments of CARLSON (1941) with Chortophaga and those of SAX (1940) and THODAY (1942) using Tradescantia show that such a relation actually exists. It has been assumed, therefore, that if the dosage is doubled $a\bar{r}$ is also doubled. This is not exactly true because as the dosage and thus $\bar{r}$ increase linearly, the coefficient a becomes smaller in approximately a linear fashion. Thus at the higher dosages the value of $a\bar{r}$ is somewhat overestimated.

There has been one assumption which has been implied but not stated in

the above considerations. This assumption is that no union of broken ends takes place during the period of treatment. If such union did occur, the empirical results would be distorted in relation to the theoretical expectations since the dosage administered to the flies was raised by lengthening the time of exposure rather than by increasing the amount of radiation per unit time. However, the evidence on this point for Drosophila seems quite clear. Neither MULLER (1940), DEMPSTER (1941), KAUFMANN (1941), nor MAKHIJANI (1945) was able to demonstrate any difference in the number of rearrangements formed when radiation was administered to sperm in such widely different ways as continuous treatment at different intensities, intermittent treatment, and continuous treatment with a delay of thirty days before mating. It is generally accepted that in Drosophila the union of broken ends takes place sometime during or after fertilization.

The final calculations leading to the theoretical dosage-frequency relation have been tabulated in table 4. It will be recalled that $S_D{}^r$, the probability of obtaining a detectable interchange from a sperm with r breaks, is obtained by summing the product $p(d) \cdot p(i_D)$ over all the possible distributions of the r breaks among the four chromosomes being followed. Similarly, $S_2{}^r$, $S_3{}^r$, and $S_4{}^r$ are the probabilities that an interchange involving two, three, or four chromosomes will be recovered from a sperm with r breaks. These figures are

TABLE 5

Comparison of the empirical and theoretical results.

APPROX. DOSAGE IN r UNITS		NO. OF SPERM WITH INTERCHANGES PER TESTED SPERM	MINIMUM NO. OF BREAKS PER TESTED SPERM	NO. OF OBSERVED INTERCHANGES	AV. MINIMUM NO. OF BREAKS PER INTERCHANGE	% INTERCHANGES INVOLVING 2, 3, AND 4 CHROMOSOMES		
						2	3	4
1000	W*	0.040	0.080	22	2.000	100.00	—	—
	C	0.042	0.084	47	2.000	100.00	—	—
	T	0.042	0.085	—	2.046	95.54	4.21	0.21
2000	W	0.097	0.203	50	2.100	90.00	10.00	—
	C	0.123	0.262	126	2.119	91.27	5.55	3.18
	T	0.137	0.289	—	2.113	89.60	9.49	0.92
3000	W	0.172	0.369	82	2.146	87.80	9.76	2.44
	C	0.256	0.562	218	2.197	82.57	15.14	2.29
	T	0.255	0.559	—	2.193	82.77	15.12	2.10
4000	C	0.353	0.805	163	2.282	74.85	22.09	3.07
	T	0.377	0.860	—	2.283	75.54	20.65	3.82

* W = warm temperature experiments, C = cold temperature experiments, T = theoretical expectation.

obtained by summing the product obtained by multiplying p(d) by $p(i_2)$, $p(i_3)$, and $p(i_4)$ respectively over all the possible distributions of the r breaks. These calculations have been carried out and the resulting probabilities are recorded in table 4. Calculations have not been made for values of r greater than 10 since there is less than one chance in 100,000 that a detectable interchange would not be recovered from a sperm with 10 breaks. The appropriate values of p(r), the Poisson distribution, for 1000, 2000, 3000, and 4000 r units

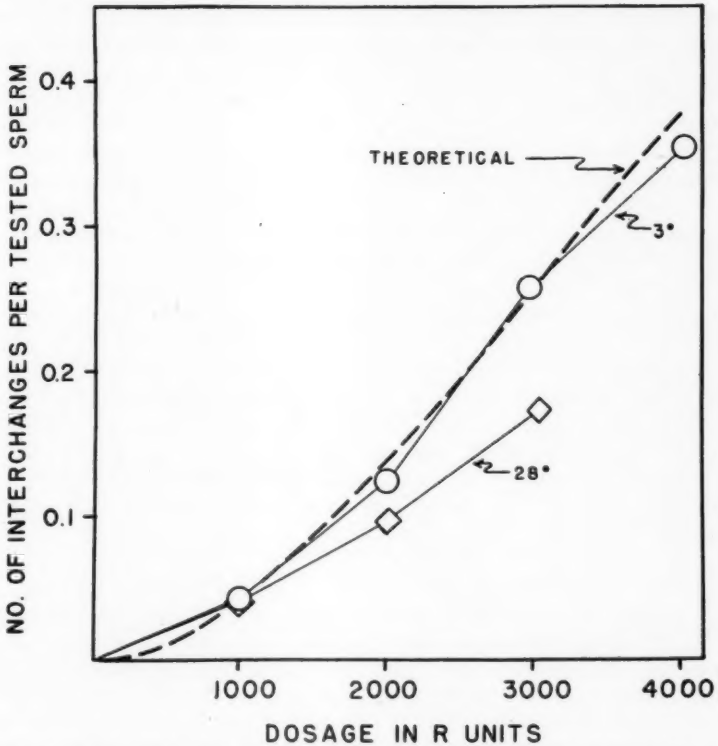

FIGURE 4. Comparison of the theoretical and empirical results. The dosage-frequency relation of the number of interchanges per tested sperm. See figure 2 for explanation of symbols.

are also tabulated in table 4. The final summations leading to the theoretical relation are presented at the bottom of this table. It will be noticed that the bold-faced figures in the first row of these summations give the probability of recovering a detectable interchange for the four dosage levels used in the experiments being reported. These values are actually the expected number of recovered interchanges per tested sperm. The summations in the next three rows of the table give the probability of obtaining an interchange involving

two, three, or four chromosomes for each of the four dosages used. The figures given in bold-faced type in the bottom row of this table are the expected minimum number of breaks per tested sperm since they are obtained by summing the product of the probability of securing a two, three, or four-chromosome interchange by the respective number of chromosomes involved.

The comparison between the theorectical expectations and the combined results of all experiments exclusive of any interchanges involving the Y chro-

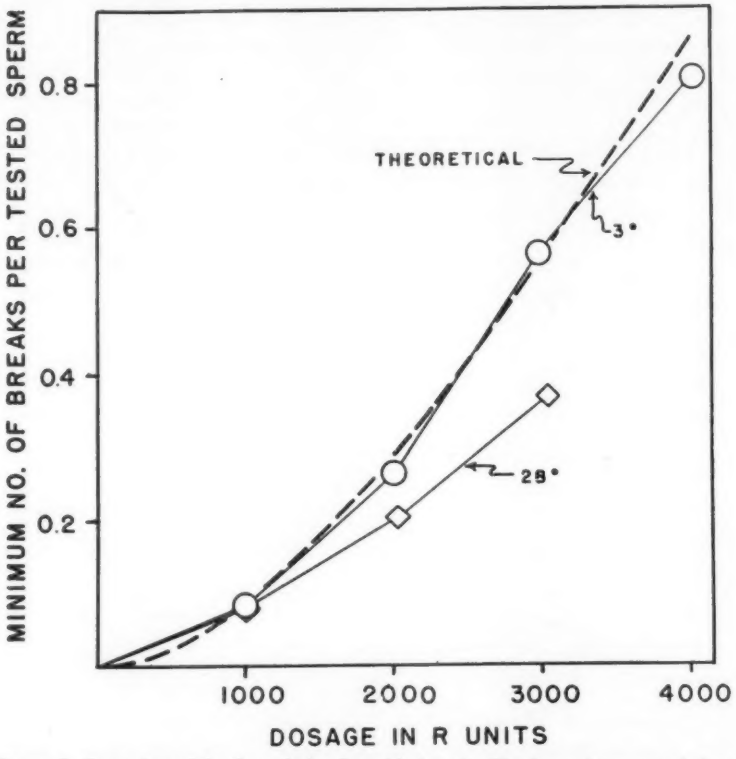

FIGURE 5. Comparison of the theoretical and empirical results. The dosage-frequency relation of the minimum number of breaks per tested sperm. See figure 2 for explanation of symbols.

mosome is made in table 5. The figures tabulated in the first two columns, relating to the number of sperm with interchanges and the minimum number of breaks, are presented graphically in figures 4 and 5 respectively. It will be noted that the results of the cold temperature experiments agree closely with the results expected from the theoretical considerations. This agreement is noteworthy since the theoretical curve probably represents a maximum curve because the assumption was made in its formulation that a broken end is as

likely to join with a broken end in another chromosome as it is to restitute. The question immediately arises as to whether this close fit is merely a coincidence or whether the chromosomes actually behave in accordance with the assumptions set forth in the theory. No definite answer can be given to this question; however, a clue is found by an examination of the relative frequencies with which translocations involving two, three, and four chromosomes are found. In the last three columns of table 5 are recorded the empirical and expected frequencies of occurrence of these three different types of interchanges. A chi-square test indicates that there is no significant difference between the expected relative frequencies and the actual values except in the case of the percent of four-chromosome interchanges in the cold temperature experiments conducted at 2000 r units ($\chi^2 = 4.8$). This close agreement provides some evidence that the assumption of random and independent union of broken ends is valid since the relative number of translocations involving three and four chromosomes should be reduced from the expectations if restitution is more likely to occur than interchange.

The second rather obvious conclusion to be drawn from the data presented in table 5 is that the results of the experiments at 28°C do not follow the expectations based on the theory. The decrease in the number of interchanges produced at the warm temperature is apparently not caused by any alteration in the randomness of the process of union since both the warm and cold temperature experiments closely follow the expected ratios with regards to the number of interchanges involving two, three, or four chromosomes. It is not surprising that a temperature change at the time of irradiation does not affect the joining of broken ends since this process takes place sometime during or after syngamy. Possible causes for the deviation of the warm temperature results will be discussed below.

THE FREQUENCY-DOSAGE RELATIONSHIP

The target theory of the biological action of ionizing radiations as applied to chromosome breakage has become a powerful working tool for present day investigators in this field. This theory implies that a single ionizing particle, either by one or more ionizations (hit) in the vicinity of particular regions of the chromosome thread (targets), is able to form a break in the chromosome. One expectation, based on the independent production of the individual particles, which follows immediately from the implications of the target theory is that the biological effect should increase linearly with the number of particles (dosage) until an appreciable number of targets have been hit. Although the determination of this expectation in Drosophila is aided by the fact that the biological effect, the breakage or potential breakage of the chromatin thread, undergoes no reverse change during the irradiation, the analysis is greatly hindered by the fact that the breaks themselves cannot be observed and thus the resulting chromosome arrangements must be used as the measure of the effect. A still further complication is introduced by the fact that only rearrangements of the chromatin are observable and in addition only those re-

arrangements which are viable in the heterozygous condition. From these considerations it is evident that the process of recombination of broken chromosome ends has of necessity been superimposed upon the series of events causing the breaks.

The lack of a complete understanding of this recombination process has made the interpretation of experimental results dealing with the relationship between dosage and the production of gross rearrangements most difficult. Although the work of OLIVER (1932), KHVOSTOVA and GAVRILOVA (1935), and CATCHESIDE (1938) were not in disagreement with a linear relation between dosage and gross chromosome exchanges, the extensive studies of BELGOVSKY (1937), KHVOSTOVA and GAVRILOVA (1938), MULLER (1938, 1940), TIMOFÉEFF-RESSOVSKY (1939), BAUER, DEMEREC, and KAUFMANN (1938), and BAUER (1939) clearly indicate that the number of rearrangements increases more rapidly than the first power of the dosage. On the basis of the latter results, MULLER (1938, 1940) proposed the "3/2 power rule". This rule means that gross rearrangements are produced in proportion to the 3/2 power of the dose. Although it is clear from numerous studies that the frequency with which these rearrangements are produced increases faster than the first power of the dose, the attachment of any great significance to the particular exponent of 1.5 seems rather dubious. It is obvious from the results presented in this investigation and from the findings of other workers, *e.g.*, BAUER *et al.* (1938), that at dosages of 4000 r units and above the slope of the frequency-dosage curve decreases giving the over-all curve a sigmoid appearance. Such a relation can hardly be described by a simple power function. Also it is apparent from the observed effect of temperature on interchange production that the exponent is a function of the temperature at the time of irradiation. And finally, it is evident from the mathematical analysis given that the particular exponent observed is related to the number of chromosomes being followed; that is, one would not expect to find the same exponent when translocations between only two chromosomes were being recovered as one would if four chromosomes were being studied.

The results of the present investigation are in accord with the experimental evidence on the dosage-frequency relation which has accumulated from the work with *melanogaster*. It is also evident that the cold temperature results are in agreement with the expectations based on the simple picture of the processes of chromosome breakage and union that is presented above. Two of the assumptions used in the formulation of this theory, namely, the random recombination of broken ends and the assumption that the union of two or more broken ends has no effect on the joining of the remaining ends, have been criticized by Fano (1941, 1943). The evidence upon which the first assumption is criticized is derived from analyses of the relative frequencies with which different types of rearrangements are recovered in the salivary gland chromosomes. Thus BAUER *et al.* (1938) and BAUER (1939) using *melanogaster*, HELFER (1941) and KOLLER and AHMED (1942) using *Drosophila pseudoobscura* found a wide discrepancy in certain cases between the recovered distribution of breaks among the chromosomes and the distribution "expected" on a

random basis. However, in determining the "expected" frequency used in these calculations no consideration is given to the possibility that restitution of breaks cause some sperm with a higher number of breaks to contribute to the class of rearrangements with fewer breaks. In spite of this omission in the calculations there may be a real discrepancy between the empirical and expected results.

Evidence that the second assumption is not wholly reliable comes indirectly with the discovery by KAUFMANN (1943) of a complex rearrangement involving at least 32 breaks. This was found in a larva whose father had received a treatment of 4000 r units of X-radiation and a subsequent exposure to infrared radiation. On the basis of the figure usually assumed for the average number of breaks per 1000 r units in Drosophila (0.4–0.6), the probability of obtaining a sperm with 32 or more breaks with 4000 r units of treatment is quite small. In addition, the probability (based on random union of broken ends) that such a sperm will give rise to a viable arrangement is about three percent (FANO 1943). Apparently FANO overcomes the obvious difficulty of explaining this case by assuming that the average number of breaks is higher than the figure stated above, and that restitution is much more likely to occur than exchange except when such an exchange happens to take place. That is, if an exchange has taken place during the process of union, this exchange imposes stresses along the chromosomes involved which cause any other breaks in these chromosomes to be less likely to restitute and thus become available for further unions. Although these suggestions appear reasonable, there is little evidence which contributes either to their support or rejection.

In view of the data just discussed, the question arises as to whether the rather close agreement between the results obtained in *virilis* and the theory can be attributed to the somewhat questionable procedure which is used in determining a value for the arbitrary constant ($a\bar{r}$) from the same experimental data to which the theoretical curve is being compared. It will be recalled that the value of $a\bar{r}$ was chosen such that only an agreement was reached at 1000 r units with regards to just the percentage of sperm with interchanges. However, with this choice it is found that the results of the cold temperature experiments agree at all dosage levels with the expectations in these three respects: (1) the percent of sperm with interchanges, (2) the minimum number of breaks per tested sperm, (3) the relative frequencies with which two, three, and four chromosomes are involved in the recovered interchanges. Furthermore, the value of $a\bar{r}$ that was chosen not only fits the results of these experiments but is in general agreement with the value postulated by other workers for the average number of breaks per 1000 r units in *melanogaster* (see LEA 1947). Thus it can be stated with some assuredness that the results of the experiments at the cold temperature are in concordance with the assumptions of random joining of broken ends and the independent union of these ends.

THE TEMPERATURE EFFECT

Soon after the discovery was made that gene mutations and chromosomal aberrations could be artificially induced, investigations were undertaken in

which the temperature of the organism receiving the radiation was altered during the period of treatment. The experimental designs which have been utilized to test for a temperature effect in Drosophila have been limited to two criteria: the rate of induction of sex-linked lethal mutations and the rate of translocation production as detected by genetic analysis.

MULLER's early experiments (1930) indicate that there is a slightly higher, but not significant, increase in the number of sex-linked lethal mutations induced in flies irradiated at 8° over a series treated at 34°C. MEDVEDEV (1935) found a significantly higher frequency of lethals produced in the flies maintained at 0° than in the ones treated at 20°C. However, TIMOFÉEFF-RESSOVSKY and ZIMMER (1939) could find no difference in the percentage of lethals induced at 10° and 35°C. This was confirmed by MULLER and MAKHIJANI (MULLER 1940) using temperatures of approximately 5° and 37°C. Finally, the recent work of KING (1947) substantiates MEDVEDEV's data since a two to three-fold increase in the mutation rate was observed in the series irradiated at 0° over the group maintained at a room temperature which fluctuated between 23° and 28°C.

The experiments which test the effect of differences in temperature on the production of translocations are apparently as contradictory as the results just cited. PAPALASHWILI (1935) found an increased number of translocations produced at 0°C over the number detected at room temperature. Similar results are reported in the more extensive tests of MICKEY (1939) who employed temperatures of about 4° and 28°C and KANELLIS (1946) who irradiated the flies at 2° and 32°C. But MULLER and MAKHIJANI (*op. cit.*) using the same temperatures as in the mutation studies, could find no increase in the number of interchanges in the cold series. It has been reported by KING (1947) that MAKHIJANI in 1944 was unable to observe a temperature effect on the production of chromosome aberrations. (The writer has not seen this paper and consequently the temperatures at which the radiation was given are not known.)

It is difficult to reconcile all these findings. MULLER (1940) has attributed the detection of a positive temperature effect to poor experimentation caused by the failure to keep the radiation from secondary radiators the same in the cold and warm series and to the inclusion in the results of sperm which had not completed the maturation divisions at the time of treatment. In certain of the experiments cited care was not taken to control these factors; however, the results reported in this paper cannot be subjected to this criticism. There is one consistent factor present in the Drosophila experiments which has in the past been overlooked. Apparently the assumption has been made that the wider the temperature range employed, the more pronounced any effect should be. This is not necessarily the case. It seems more than a passing coincidence that in all of the experiments reporting a positive effect of temperature, the cold temperature used varied from 0° to 4°C and the warm temperature from 20° to 32°C. While in all of the experiments indicating no effect, the two temperature ranges used varied from 5° to 10°C and from 34°

to 37°C. It appears, therefore, that an increase in the induction rate of translocations accompanies a change in temperature either above or below room temperature. This increase is similar to the result PLOUGH (1917) obtained while studying the effect of temperature on the frequency of crossing over in *melanogaster*. He found that at 13° and 31°C the percent of crossing over is almost three times greater than at 27°C. In order to prove that such an increase actually occurs, the mutation and interchange rate should be measured in comparable experiments conducted at 28° and 36°C.

The question now arises as to whether temperature affects the breakage or the joining process. In Drosophila sperm no union of broken chromosome ends takes place until syngamy, thus these two processes are separated in time. Although it is difficult to see how a temperature change at the time of irradiation could affect the course of union at fertilization, it is equally perplexing to see how it could alter the process of chromosome breakage since the physical action of X-rays is independent of temperature in the range utilized (LEA 1947). The union of broken ends is not delayed in Tradescantia and it has been postulated that the decrease in the number of chromosomal aberrations observed when the temperature is raised during X-radiation is caused by an alteration in the process of union (SAX and ENZMANN 1939; FABERGÉ 1940; CATCHESIDE, LEA and THODAY 1946; and SAX 1947). The recent work of FABERGÉ (1948) casts doubt on this interpretation since he found a temperature effect on the number of aberrations when the radiation and temperature change were administered in about one-third of the average time that a break remains free. When viewed in this light, it is not surprising that MULLER (1940) and KAUFMANN (1946b) were unable to show an effect of different temperatures on Drosophila females fertilized by irradiated sperm; that is, temperature differences at the time of union. FABERGÉ also reports that a change in temperature in either direction immediately preceding treatment considerably increased the number of aberrations. He states that a possible explanation of this behavior may lie in the fact that thermal diffusion caused by the temperature gradient moves the chromosome threads, thus making restitution less likely. It is tempting to draw an analogy to the Drosophila experiments which apparently show a rise in the frequency of translocations at temperatures above and below room temperature; however, in many of these experiments the flies were placed in the cold chamber a sufficient length of time before treatment to neutralize any temperature gradient.

The possibility also exists that the temperature effect is not caused by the temperature *per se* but rather by its effect on some cellular process such as respiration. Recently, THODAY and REED (1947) demonstrated a three-fold increase in the number of anaphases showing chromosome aberrations when oxygen instead of nitrogen was bubbled for a short time before and after X-ray treatment through a cell of water containing *Vicia faba* roots. Cytological examinations were made at intervals after treatment varying from a few to 48 hours. The fact that the increase was consistently evident at these various times would seem to indicate that the breakage, not the union process

was being affected. If the activity of a fly at a particular temperature is taken as a rough index of the rate of respiration, the work of CHADWICK (1939) on the frequency of wing beat of Drosophila would indicate that the respiration rate is greatest at a temperature of about 29°, being much lower at 8° and 36°C. Thus at 29°C there would presumably exist the greatest gradient in oxygen tension within the tissues and consequently a lowering of the oxygen concentration in the testis. Viewed in this light, the temperature effect in Drosophila falls into line with the bean root experiments; however, the mechanism whereby the oxygen tension affects chromosome breakage is unknown.

It is evident from the considerations discussed above that, although the method whereby heat at the time of X-ray treatment affects chromosome breakage is still undetermined, there seems no reason to doubt that the temperature effect on irradiated Drosophila chromosomes is a reality. The evidence obtained from the *virilis* experiments indicates that the decrease at the warm temperature in the number of sperm with interchanges is not associated with the process of union of the broken chromosome ends, since the relative number of translocations involving two, three, and four chromosomes agrees with the cold temperature results and with the expectation based on the assumption of random and independent union. It seems most probable that a temperature difference at the time of irradiation alters, directly or indirectly, the process of chromosome breakage.

SUMMARY

Males of *Drosophila virilis* were treated with 1000, 2000, 3000, and 4000 r units of X-radiation. During irradiation the flies were kept at a temperature of either $3 \pm 1°$ or $28 \pm 1°$C in comparable experiments. Any major chromosome interchanges induced in the sperm of these males involving the four main autosomes and the Y chromosome were recovered by genetic analysis of their F_2 offspring.

The relationship between dosage and translocation production as determined by these experiments is found to be similar to the relationship previously reported in *D. melanogaster*. The percent of sperm with interchanges increases more rapidly than the first power of the dose, but gradually slopes off around 4000 r units giving a sigmoid-shaped curve. A somewhat similar relation is found to exist between dosage and the minimum number of breaks induced in the sperm. This increase in the number of breaks is not caused solely by a rise in the number of simple translocations recovered, but is in part caused by the fact that, per individual sperm, more chromosomes are involved in the interchanges induced at the higher dosages.

The frequency-dosage relation is not the same in the warm and the cold temperature series. A significant lowering of the rate of induced interchanges is observed at the higher dosage levels when the flies are maintained at the warm temperature during treatment. This finding confirms the results of other investigators who used warm temperatures of around 25°C and observed a temperature effect. It has no direct bearing on the negative results reported by workers who employed a warm temperature of approximately 36°C.

An analysis of the chromosomes involved in over 800 of the induced trans-

locations indicates that the four major autosomes are about equally likely to participate in any interchange, but the frequency with which the Y chromosome is involved is less than half as great as any one of the autosomes. This indicates that the four major autosomes are about equally likely to be broken by the ionizing radiations.

In order to evaluate the significance of the observed frequency-dosage relation, the mathematical expectations based on a simple theory of chromosome breakage and union are derived. The formulation of this theory involves the following main assumptions:

1. The number of breaks per sperm follows the Poisson distribution.
2. The mean number of breaks per sperm increases linearly with dosage.
3. Each of the major autosomes is equally likely to be broken.
4. The broken chromosome ends unite at random to form the resulting arrangements.

A comparison between the theoretical expectations and the empirical results (exclusive of interchanges involving the Y chromosome) brings to light several pertinent facts concerning the processes of chromosome breakage and union in *D. virilis*. The cold temperature experiments follow very closely at all dosage levels the expectations not only in respect to the percent of sperm with interchanges, but also in regards to the minimum number of recovered breaks. Moreover, a close agreement is evident between the expected and actual frequencies with which interchanges involving two, three, and four chromosomes occur. On the basis of this result it is apparent that restitution of broken chromosome ends is no more likely to occur than recombination, thus lending support to the assumption that the union process is a random and independent joining of broken ends. The results of the warm temperature experiments fall below the expectations based on the theory. This decrease in the number of recovered interchanges cannot be attributed to a disturbance in the process of joining of the chromosome ends which would increase the chance of restitution, since the relative frequencies with which two, three, and four chromosomes are involved in an interchange again agree with the expectation based on the assumption of random union. Although it is not known whether the temperature effect is caused by a direct action of heat on the chromosomes being irradiated, it seems likely that the ultimate effect of heat alters the mechanisms involved in the breakage of the chromosome thread.

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SELECTION FOR SMALL AND LARGE BODY SIZE IN THE HOUSE MOUSE*[1]

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INTRODUCTION

THE animal breeder's main and typical problems concern economic characters, such as meat or wool production, yield and fat content of milk, number and size of eggs. These are complex, quantitative characters, multifactorial and strongly influenced by environmental conditions. The great majority of the fundamental anatomical, physiological and taxonomic characters, and basic processes like growth and reproduction, are of this class. For man to change and guide improvements in such quantitative characters usually requires continuous, gradual selection of large numbers of individuals over a long series of generations. This is practically a return to DARWIN's artificial selection by slow increase of small variations, which must be credited with the principal improvement of domestic animals over past centuries, and appears to be the chief hope of further improvements to come.

The main object of this paper is to describe further the primary and secondary effects of a form of mass selection in an experiment designed to create for laboratory uses from one foundation stock both a small and large race of house mice. Progress reports (MACARTHUR 1944, MACARTHUR and CHIASSON 1945) gave the procedures and results for the first eight generations of the selections. The series is now extended to cover at least 21 generations, averaging three a year over seven years. The environment was kept similar for all the mice at any one time by having them share the same quarters, feed, care, *etc.*, except that sibs were fed and reared by their own mothers. Maternal care itself varied greatly.

SOURCES OF VARIATION

Genetic variations, the necessary raw materials for the changes in size, were presumably supplied by numerous multiple factors in nearly all chromosomes and segments, partly neutralizing each other's effects, some promoting, some retarding general growth. The field of variation was probably widened by intercrossing six inbred strains of mice of average size but of various colors. (GOODALE's (1938) successful size increases in albino mice began without

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[1] Based on the paper, "What Mass Selection Can Do" given at Chicago, December 31, 1947, in the Symposium, "Methods for the Improvement of Farm Animals" at a joint session of the GENETICS SOCIETY OF AMERICA and the AMERICAN SOCIETY OF ZOOLOGISTS.

[2] Formerly of Department of Zoology, UNIVERSITY OF TORONTO, which generously supported this research.

much prior hybridization.) It would be expected that fresh new mendelian combinations and interactions of genes would be made rapidly available by segregation and assortment of chromosomes with excess plus or minus balance; and that further cryptic variation would gradually be released by crossovers within chromosomes and such new mutations as may occur. The job was to re-shuffle the size genes, and unpack and reorganize the chromosomes to bring together as many plus genes as possible in the large race, and collect the minus genes in the small race.

TYPE OF SELECTION

It seems misleading to draw sharp contrasts between mass selection and pedigree culture, genotypic selection or discontinuous selection. The traditional usage tends often to confuse the separate problems of accuracy of judging (with or without measurements), thoroughness of pedigreeing (with both, one or neither of the individual parents known), criteria of selection (by performance of the individual, of sibs, parents, progeny, etc.) and systems of mating (random or non-random breeding; as one population, or subdivided into groups). It is easily possible to modernize the cruder old form of mass selection, by adopting more quantitative methods, by keeping pedigrees, and by recognizing that inheritance even of a continuous variable, is particulate and that genotypic selection is superior to phenotypic. Mass selection, proceeding on a wide front, broad-based to avoid ill effects of inbreeding, could even be considered the most generalized form, of which group, line, family, pair and individual selection are special and, often very useful, limiting cases.

In these experiments all mice were earmarked, sexed, and individually weighed. Selections were made by comparing the growth in weight of individuals and sibships with their parents and sometimes with their progeny. Pairings among the large, for instance, were mainly at random within a single closed population, each male with several females, to pool and recombine and concentrate as far as possible the plus size genes within the selected population. Extra litters were bred from pairs proving their transmitting ability by begetting a first litter of promising body size; such reproductive selection multiplied favorable genotypes and effective growth-promoting (or growth-retarding) interactions. It was intended that only the most helpful genotypic combinations were put back into the pool.

CHANGES IN THE PRIMARY CHARACTER, BODY SIZE

From the first the body size increased noticeably in the large and diminished in the small (figure 1). The trends are quite like those made familiar in WINTER'S Illinois maize experiment, PEARL'S selection for egg number in fowls, CASTLE'S for amount of white or dark in hooded rats, several which raised or lowered resistance to specific diseases, and many others with laboratory and domestic animals. Methods used in the several experiments differ chiefly in details of choosing the foundation stock, in the pedigreeing of one or of both parents, in the numbers raised, in the selection differentials or in the heritability of the characters.

The results in our case suggest what can be done by mass selection in general, and particularly if one is interested in the age-old problem of more loaves and larger fishes.

With a little patience one can photograph the living mice. After 14 generations of mass selection, females two months old of the small race averaged less than 12 grams; those of the large race nearly 31 grams (figure 2). This difference, 19 grams, was increased further to 23 grams by seven more generations of the mass selection. Males are about 20 percent larger than females. At 60 days of age the large race males were 2.6 times as heavy as the small in the 14th generation, and 3.3 times as heavy in the 21st generation. Such size

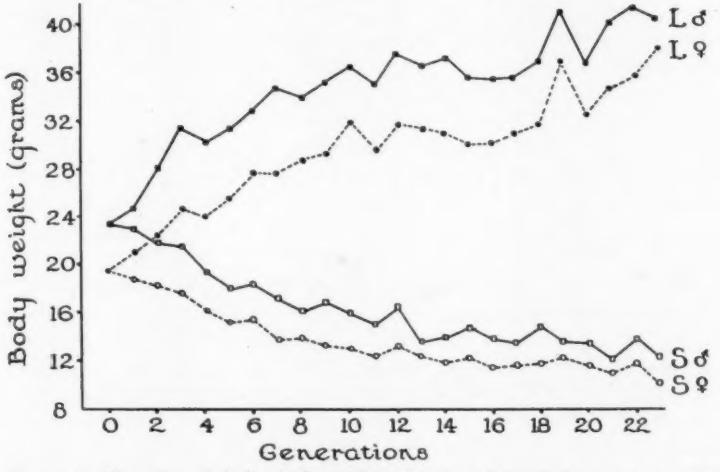

FIGURE 1. Divergence of 60-day body weights of male and female house mice, resulting from genotype mass selection continued 23 generations. Plus selection above; minus selection below.

differences are maintained to maturity and old age (figure 3). These products of mass selection recall the size range from bantams to Jersey Giants, ponies to Percherons, Chihuahuas to Great Danes or Mastiffs, and even between pigmy Negritos and large races of men. It seems not unreasonable to assume that comparable size changes could be made by selection in other species of domestic birds and mammals where such size differences do not exist. One may note here that the small race mice are excitable, "of nervous temperament," a phrase often used to describe the small Mediterranean breed of fowls, small breeds of cattle, *etc.* A tendency to obesity in the large might be avoided by selecting on the basis of measurements rather than weights.

The weight contrasts developed by the plus and minus selections are shown by a graphic method (figure 4) to help visualize the process. The ranges ceased early to overlap, so that in both sexes the smallest of the large race are distinctly larger than the largest of the small. The weight differences became

increasingly evident and significant (table 1) throughout the experiment, but about 60 percent of the total change occurred in the first third of the work. Apparently the early selections picked out the gene and chromosome combinations and interactions with major size effects, and the later selections had to depend more on those with minor size effects and new cross-overs.

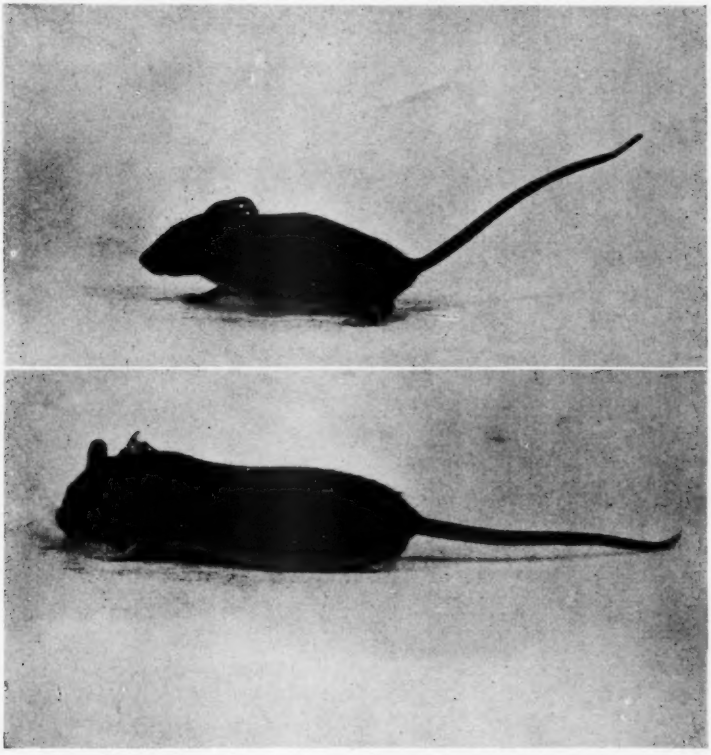

FIGURE 2. 60-day old females of the small and large races in the 14th generation of selection. Note also the differences in coat color, alertness and size of ears and tail.

Other graphs (figure 5) show well the amount of change produced by comparing weight distributions for the initial stock and the selected races. The noticeable asymmetry in most of the distributions suggests that the metric scale needs to be altered to normalize the curves. The range of absolute sizes is, of course, narrow in the small race, wide in the large.

One may quote with approval an apt statement by FISHER (1931), that "natural selection is a mechanism for generating improbability of a high order". Artificial mass selection also generates improbability of a very high

order, since it makes sizes beyond the extreme tails of the original frequency distribution the very center and average of the selected populations. In fact extremes such as would and perhaps could never occur in the first generation become common and usual. Under selection pressure minus genes more and more collect in the small, plus genes in the large strain. Two-way selection deliberately culls average specimens and draws non-random samples, the breeding of which makes further and more extreme deviations likely. In terms

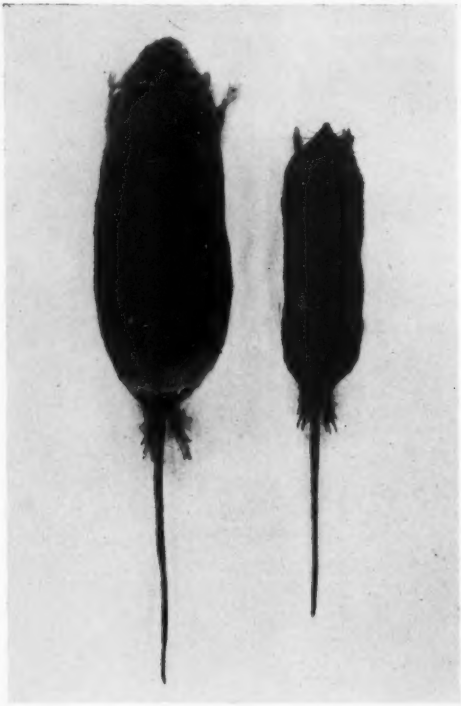

FIGURE 3. Three month old males of the 23rd generation; weight of the small 16.2 gms, of the large 58.0 gms.

of generation O the range between S-21 and L-21 means is 10.9 standard deviations. The chance of drawing from the foundation stock an individual male as small as the S-21 mean would be of the order of 1 in 100,000; the chance of picking an average L-21 male would occur only about once in a population of a thousand million; here again the skewness is obvious. The selection process resembles valve action, permitting flow only in a preferred direction. Each step makes possible the next forward step. These steps depending as they do on a particular succession of chance cross-overs, matings,

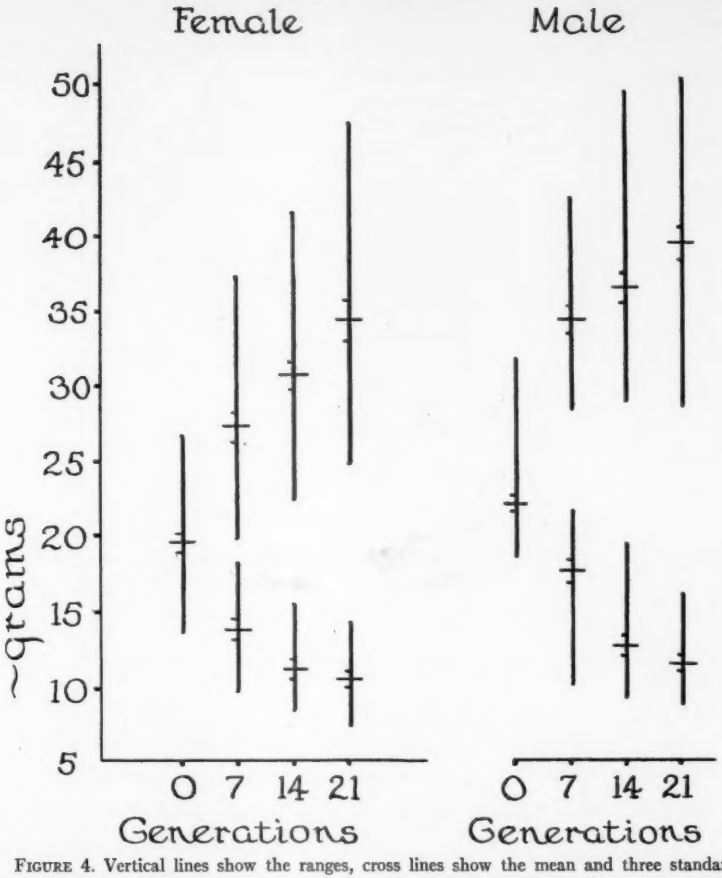

FIGURE 4. Vertical lines show the ranges, cross lines show the mean and three standard errors of the mean for the populations at the beginning and after 7, 14 and 21 generations of the mass selection.

TABLE 1

Divergence of small and large races of mice in grams body weight at 60 days, and significance of the inter-racial differences.

GENERATION	FEMALES		MALES	
	GRAMS	STANDARD ERRORS	GRAMS	STANDARD ERRORS
0	0		0	
7	13.95	29.4	17.44	47.8
14	19.02	51.6	22.86	52.4
21	23.67	55.5	27.88	54.7

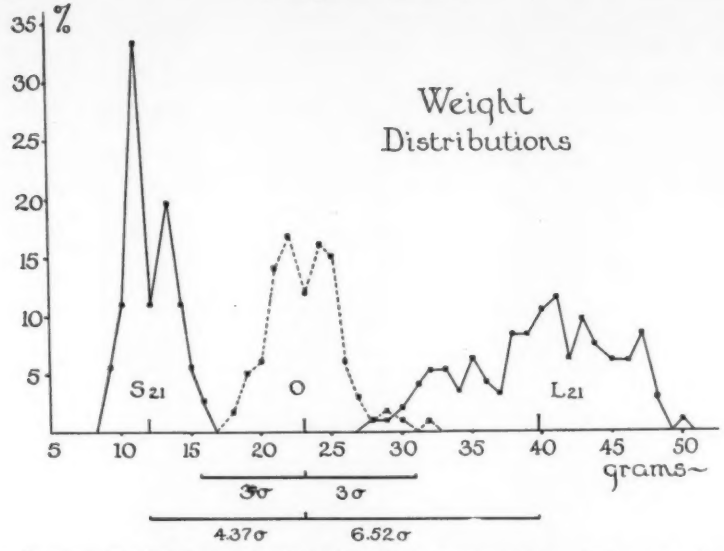

FIGURE 5. The distribution of male 60-days weights for the initial stock (0, at center), and after 21 generations of minus selections (S-21, at left), and of plus selections (L-21, at right). The standard deviations below refer to the foundation stock 0.

combinations, *etc.* must be in any precise sense, largely unrepeatable and irreversible.

As the mean weights increase the standard deviations, of course, also increase (table 2). But it will be noticed that the coefficients of variability con-

TABLE 2

Sizes (body weights in grams at 60 days) of male and female house mice from the unselected foundation stock (generation 0), and from the small and large races after 7, 14 and 21 generations of plus and minus selection.

GENERATION	MALE			FEMALE		
	MEAN WEIGHT	STANDARD DEVIATION	COEFFICIENT OF VARIABILITY	MEAN WEIGHT	STANDARD DEVIATION	COEFFICIENT OF VARIABILITY
L-21	39.85± .47	5.10	12.80	34.46± .49	5.12	14.86
L-14	36.79± .37	3.66	9.95	30.71± .31	3.29	10.71
L- 7	34.69± .37	3.59	10.34	27.51± .39	3.80	13.81
0	23.16± .26	2.56	11.01	19.51± .25	2.65	13.52
S- 7	17.25± .30	2.61	15.13	13.56± .27	2.34	17.36
S-14	13.93± .23	2.13	15.29	11.69± .20	1.74	14.89
S-21	11.97± .28	1.71	14.29	10.79± .26	1.47	13.62

tinue throughout at about the same percentage level undiminished by selection. Apparently the small and large races are not becoming more uniform in weight, though genetic variability is theoretically steadily lessened (compare table 6). This suggests that when genetic variation is reduced, plastic modifications increase to a compensating degree. It is as though the mice develop natural phenocopies of genetic weight differences; and recalls the old principle of organic selection.

TABLE 3

Differences in grams of mean body weights (both sexes) of the small and large races at birth, 30 days and 60 days, and the increase during the first and second months.

	BIRTH-FED	30 DAYS	60 DAYS	MATURE	INCREASE		
					0–30 DAYS	30–60 DAYS	2–6 MONTHS
S-21	1.04	10.81	11.44	17.27	10.4×	1.08×	1.51×
L-21	1.44	22.95	37.16	56.95	15.8×	1.57×	1.54×
L-21/S-21	1.38	2.12	3.24	3.30			

We may ask how these marked size differences came about in ontogeny. Our own data are chiefly for the postnatal period. Taking GREGORY and CASTLE'S (1931) work with rabbits as a cue about the prenatal period, the eggs of small and large mothers are probably still of equal size (perhaps about 0.06 mm in diameter). The suggestion is that, starting without initial differences in capital, the growth of the large race embryo is considerably faster than in the small race embryo (table 3). Birth-fed young are already 38 percent heavier in the large mice. In the first month of postnatal growth, the small race young increase about 10 times, the large race young more than 15 times in weight. The same difference in rate is found also in the second month. The later gains, from two to six months, however, are nearly equal on a percentage basis (151× for small, and 154× for large). At maturity the large are some 3.3 times larger. As we interpret the data, growth during the first 60 days is about half again as rapid in the large mice. That is the gist of the change induced by the selections.

It is as though two racers start from scratch and run as far as they can in the same time. One of them by getting off to a quick start and by showing an early burst of speed, takes a long lead, more than holds it through the race, and at the end has gone three times as far.

Since there is a high correlation between birth weight and later mature weights an early estimate of potential meat production seems practicable. Also since early growth is so important, it might be worthwhile to try selecting directly for birth weight itself, making due allowances for such influences as age of mother and number in litter.

We may also ask whether it is as easy to decrease as to increase body size

TABLE 4

Estimates of the efficiency of the minus and plus selections vary according to scales of measurement used.

DIFFERENCE IN	$O-S_{21}$		$O-L_{21}$	
	MALES	FEMALES	MALES	FEMALES
grams	−11.19	− 8.72	+16.71	+14.95
percent	93.5	80.8	72.2	76.6
logs	.2866	.2572	.2560	.2471

by selection. The answer (table 4) varies with the metric scale adopted. In absolute grams or pounds the gains by plus selection much exceed the losses by minus selection; if mice of the small race had lost as many grams as the large ones gained, their weight would be less than three or four grams, and must soon approach the zero point. The change in the small is, however, greater by a log scale, or in percentage by which a greater value exceeds a lesser.

TABLE 5

Deviations (in σ) from their population means of sires and dams chosen for breeding.

GENERATIONS	SMALL RACE		LARGE RACE	
	SIRES	DAMS	SIRES	DAMS
0– 7	1.71	1.04	2.31	1.41
8–14	.66	.48	1.89	1.41
15–21	1.07	.41	1.60	.63
0–21	1.18	.69	1.80	1.01

We planned to try equally hard to lower and to raise the body size. It turns out in retrospect, however (table 5), that there was actually a higher selection differential in the large than in the small. This may have been due to the more numerous and larger litters from which to choose élite for plus selection.

TABLE 6

Measures of success in selecting for extremes of body size at 60 days.

GENERATIONS	PAIRS PRODUCING YOUNG	DIVERGENCE GRAMS PER PAIR	PROGENY RAISED	DIVERGENCE GRAMS PER 100 YOUNG	DIVERGENCE HELD / DIVERGENCE SOUGHT %
0– 7	201	.077	2205	1.41	23.78
8–14	260	.021	2371	.45	12.28
15–21	326	.015	2474	.40	11.44
0–21	789	.033	7050	.73	16.96

TABLE 7

Relation of number of young per litter to weight of young at birth, and weight of males at 30 days and 60 days in large race mice (L-21).

LITTER SIZE	MEAN WEIGHT (GRAMS) AT		
	BIRTH	30 DAYS	60 DAYS
4– 7	1.58	26.87	43.99
8– 9	1.51	22.22	39.43
10–11	1.52	21.78	39.11
12–14	1.38	23.76	38.90

Through the experiment the average generation consisted of 37 pairs successfully bred and 336 young reared to 60 days (table 6). The response to selection effort was roughly measured in terms of the divergence per pair bred, or per 100 young raised, or by dividing successes by pretensions (last column). This last is an estimate of heritability; it matches the superiority of the young with the superiority (over the parental generation) of the chosen

TABLE 8

The effect of foster mothers on growth of young exchanged between S-18 and L-18 mothers.

STOCK	FOSTERED BY	WEIGHT (GRAMS)		
		BIRTH-FED	30 DAYS	60 DAYS
S-18	Small	1.07	10.21	13.05
S-18	Large	1.17	13.26	15.89
L-18	Small	1.47	21.34	35.40
L-18	Large	1.42	19.61	34.11

parents. By all three methods it is clear that the selection has achieved diminishing returns as the experiment proceeded. Heritability has declined from about 25 to 10 percent. The size variation is still there, but less of it is genetic. Incidentally, both we and GOODALE are currently troubled with some infertility of matings. Overcoming this, we may expect some further returns until unknown limits are reached. The small race is not yet quite as small as the closely related wild Asiatic species, *Mus bactrianus*.

If there are size alleles, or an excess plus or minus balance, in each of the twenty pairs of mouse chromosomes, it would require a population exceeding a million million merely to obtain in one generation the full free assortment of the chromosomes. It is, of course, impossible to obtain simultaneously all the various cross-overs which would yield plus or minus imbalance. With a necessarily small population great numbers of promising potential combinations and interactions are continually discarded untested, and irretrievably lost. If such an experiment with mice or any larger domestic animal could be carried

out several times or cooperatively in several places, the end products, doubtless differing in genotypes, due to different drift and different selections, could be interbred, both to improve fertility and to make possible new progress by size selection. At this point mass selection would turn into group selection and the intercrossing of temporarily isolated groups could secure for the breeder the equivalent of the rapid evolutionary advances predicted by WRIGHT.

The mother's genetic influence on the birth weight of her young is evident. A relation also exists between the number of young born per litter and their postnatal growth (table 7). Where young are fewer in a litter they have a weight advantage at birth which lasts to 30 and even to 60 days. This could nullify some plus selection and tend to reduce litter size in the large race.

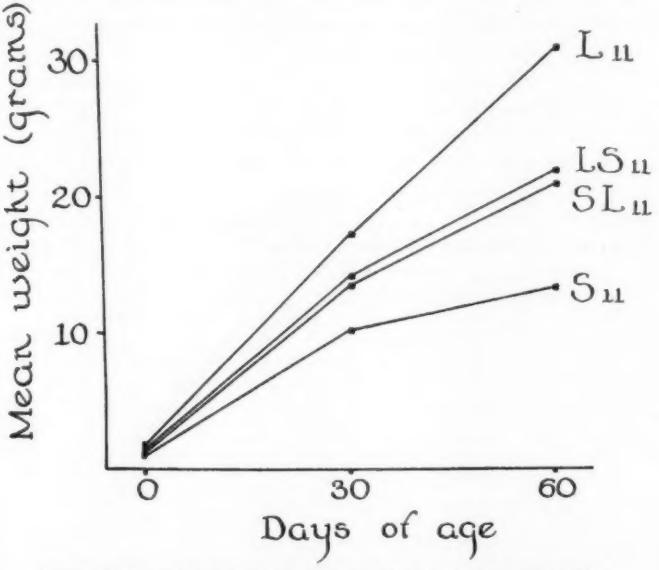

FIGURE 6. Growth of mice of the small race (S-11), the large race (L-11), and of their hybrids (LS from large mother and small father).

The attention recently drawn to milk factors or agents lead to a perhaps unnecessary test of growth when new-born young were exchanged and nursed and reared by foster mothers, a large mother feeding small race young (table 8). The "small" young grew to small size even with a large mother to foster them; the "large" young grew large on the small mother's milk. We can only conclude that if an elephant baby grows by pounds daily, it is not because it was fed on elephant's milk, but because it was an elephant's child. It is the grower's genotype that counts chiefly here, as the limiting factor in growth.

To determine whether any isolation was developing between the small and large races, reciprocal crosses were made in generation 11 (figure 6) and again

in generation 17 (figure 7). The races interbred readily, and the few hybrids and F_2 approximated an intermediate size. The young from the cross with the large mother were 11 percent heavier at birth, 2 percent heavier at 30 days, and about equal in size at 60 days and thereafter. There is no clear indication in these small samples of any matroclinal or cytoplasmic inheritance, or of any long persistence of the nutritional advantage of being fed pre- or postnatally by a large mother. This problem can be best approached by the double mating method.

It is planned ultimately to learn more about what selection can do by studying these crosses in adequate numbers. The data from selection and from hybridization should complement; and alternately taking the races apart, and bringing them together a few times, should help us to understand both the action of selection and the nature of size inheritance.

CHANGES IN SECONDARY OR CORRELATED CHARACTERS

There was little surprise at the changes wrought in body size, unless it be in their speed and magnitude. More unexpected were the wholly unsought and unintentional changes in a number of other characters that followed in the wake of the size selections. It early dawned on all who worked with the mice that the small and large races were coming to differ in coat colors, in temperament, in proportion of parts, and in litter size. We should emphasize that at no time was selection directed on any of these secondary characters.

The by-products of mass-selection deserve attention in their own right; the by-products sometimes might have greater value or interest than the main product. It is likely that these, similar, or countless other correlated characters are to be anticipated in any such experiment with other species of animals. Pull one string and you may expect distant parts to move, because of internal hidden connections.

Examples may be given which throw some light on what Darwin called "the mysterious laws of correlation." The correlations we happened to notice affect all kinds of characters, and are caused, as we read the data, by a variety of mechanisms (drift, pleiotropy, relative growth, linkage, etc.). The associations of characters may be spurious (as in drift) or real; ephemeral, as in linkage, or enduring as in pleiotropy.

(1) A few of the large mice, none of the small, are albinos; a few of the small, none of the large, are spotted. These color genes are not known to affect growth rates, but small populations by mere chance losses among neutral genes, will gradually "drift" apart in unpredictable ways not controlled by the selections.

(2) In certain coat colors the mouse races differ sharply, by a true correlating mechanism (pleiotropy). The gene pairs, black-brown, *Bb*, and deep-dilute, *Dd*, are or act as if they are, both color and size genes. The dominants, *B* and *D*, known (CASTLE 1941) to retard general body growth are found in all the small mice; their alleles, *b* and *d*, which accelerate growth are characteristic of the large race.

(3) The small and large races came to differ also in the proportions of their chief appendages to total body dimensions. The small mice have comparatively large ears, feet and tail (figure 2). But as the body grows, the growth of appendages does not keep pace. The relative growth, or allometric constants are such that selection for greater body size will automatically produce mice with relatively smaller appendages, and this without change of genes specifically controlling appendage size (See MacARTHUR 1944, figs. 1–2 and MacARTHUR and CHIASSON 1945, tables 1 and 4).

(4) When bodies increase in size, their surface areas increase more slowly than their volumes or weights. Small animals therefore present relatively large exposed surfaces, from which comparatively greater heat losses occur. One might possibly expect this to affect general activity and metabolic levels as calorimetric tests have shown. At any rate the small mice are more active, "wild," and nervous; the large ones docile and phlegmatic. The large race

TABLE 9

Counts of corpora lutea and fetuses in generations 12 and 13 of small and large race females bred with own and other race males.

MATINGS ♀×♂	DISTRIBUTION OF NUMBERS OF CORPORA LUTEA																		CORPORA LUTEA (MEAN)	FETUSES (MEAN)
	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	
S S	1	2	1		1	1														7.2	5.3
S L	2		5	3	2	2														8.1	5.5
L S							3	1	3	3	1	4	2	1	1		1			15.2	10.4
L L								3	1	4		1	3	3					1	14.1	10.5

mice are also faster growing and more economical in gains from feed taken. A student is looking into various physiological differences, and trying to measure body heat activity, basal metabolism, and to make some endocrine assays for thyroid and pituitary glands.

(5) It turns out also that there is an important tie between growth and reproduction. The small race mice develop slowly, breed a little later, and produce fewer young per litter. The large race are earlier and more prolific breeders. Litter size is positively correlated with body size (figures 8,9,10 and table 9). The correlating mechanism may operate through the endocrines (gonadotropin), or through relative growth, a large body having large ovaries, releasing more ova to produce more young in a litter.

(6) Linkage and crossing over of genes is almost certain to cause some correlated changes. Extreme selection for body size involves much sorting and reorganization of chromosomes. Multiple factors affecting two or more different characters are probably interspersed in some segments of various chromosomes. A selection for size could naturally cause a wide-spread sympathetic disturbance in many other quantitative characters, due to genic imbalance. If selection is pushed too far too fast, the organism may well be left without adequate buffer protection. There is indication that fertility is so upset. The fertility imbalance could be temporary and correctable, but mass selec-

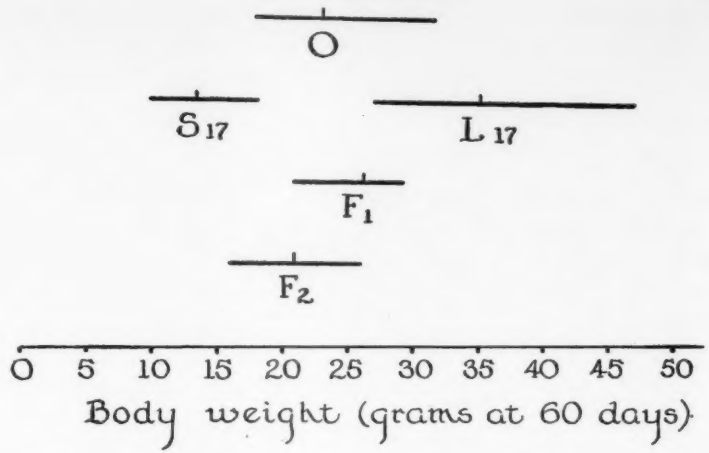

FIGURE 7. Range and mean of 60-day body weights compared in the mice of the foundation stock, the two selected races, and their F_1 and F_2 hybrids (Large ♀ ×small ♂).

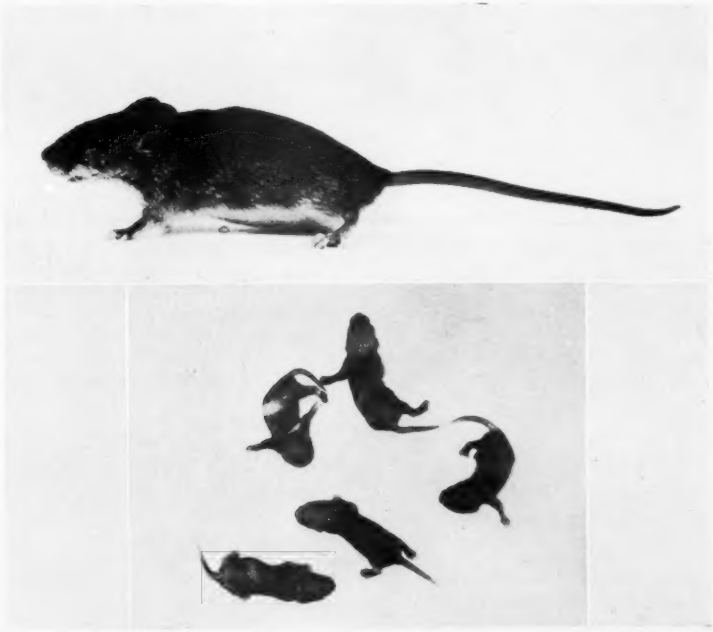

FIGURE 8. A small race (S-14) mother and a typical first litter.

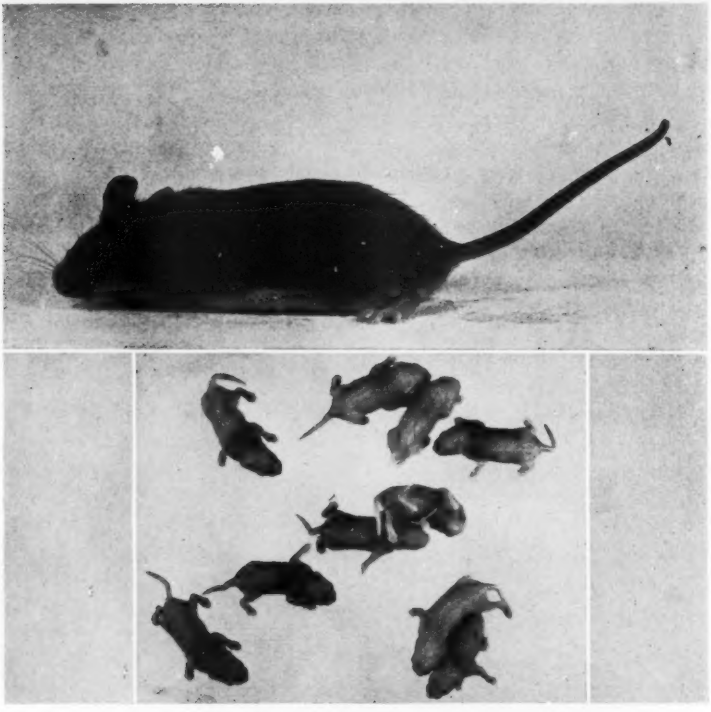

FIGURE 9. A large race mother (L-14) and a typical first litter.

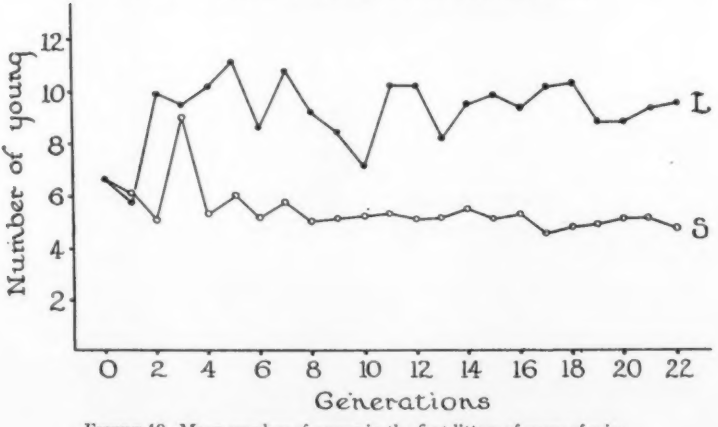

FIGURE 10. Mean number of young in the first litters of races of mice selected only for body size (weight at 60 days).

tion for one character may well necessitate or invite secondary selections for other characters.

It is unnecessary to close with any formal summary. The practical specialist is better able to judge whether the findings reported may find application with other species and other characters. It is conceivable that small races with relatively large surfaces would have some advantage for production of wool, pelts or hides; or in areas where forage is scanty. On the whole, many of the qualities commonly valued in farm animals are closely inter-related in a size complex, including large body size, faster growth, economical gains from feed, tameness in domestication, and early, rapid and efficient reproduction. Greater milk yield is almost necessarily also involved.

It may also be suggested that, since mammalogists and ornithologists have found that races of warm-blooded animals from high latitudes and altitudes are commonly of larger body size with smaller appendages and larger litters, then, possibly, by the principle of "ecological indicators," a type rather like the large race may be naturally "preadapted" to life in colder climates.

Looking back over the experiment, we should be inclined to stress: first, how strikingly an organism may be remodelled by a brief term of mass selection in such fundamental characters as its growth and reproduction; secondly and chiefly, the revealing glimpse it has given of some of the integrating mechanisms which tie together a group of characters usually studied one at a time as if independently inherited.

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THE INDUCTION OF MUTATIONS IN PARAMECIUM AURELIA BY BETA RADIATION

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FOR the study of mutation induction by radiation *Paramecium aurelia* has some advantages. The animals can be made completely homozygous by the process of autogamy (SONNEBORN 1939), and so any mutations produced in the diploid micronuclei can be brought to light easily. Lethal mutations can be kept in the heterozygous state and so subject to further genetic analysis. In addition, there is available in fission rate a character which would be expected to be affected by many different genes. Thus, in theory at least, it should be possible to obtain a measure of the overall mutation rate including mutations with individually small effects.

Counterbalancing these advantages, certain drawbacks must be mentioned. The detection of chromosomal aberrations by cytological methods is rendered difficult if not impossible by the small size of the nuclei and chromosomes. There are few known genes in Paramecium so that a refined genetic analysis of the individual changes would be difficult. Finally, each exautogamous clone, the equivalent of an individual in higher organisms, must be kept in a separate container thus considerably increasing the labor and decreasing the numbers that can be handled.

Nevertheless, it has been possible to carry out an analysis of the induction of mutations by beta radiation, to obtain some information about the kind of mutational changes involved, and to make calculations, on the basis of certain theoretical considerations, of the number of mutations induced. In addition to the information which this organism can give about the mutation process, such studies form a necessary background for a general investigation of the effects of radiation upon Paramecium, especially effects on the cytoplasm, for the study of which Paramecium is unusually well suited.

KIMBALL (1947) has given a preliminary account of the work done with an external source of beta radiation using stock 90 of variety 1. POWERS (1948) in an independent investigation has reported somewhat similar results using stock 51 of variety 4 and beta emitters in the culture medium. For solutions of equal activity, he found that P^{32} was more effective in inducing mutational changes than $Sr^{89,90}$, and Y^{90}. The exact interpretation to put upon this result is not clear, but on further analysis it may well yield important information about the mutation process.

[1] Valuable assistance was given in the planning and carrying out of the experiments by NENITA GAITHER, ROMA GOULD EISENSTARK, and MARGARET SLUSOER GRIESS.

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MATERIALS AMD METHODS

The work to be reported was carried out with stock 90 of variety 1 which was obtained originally from PROFESSOR T. M. SONNEBORN.

The procedure used to detect mutational changes was as follows. A number of animals of a single clone were exposed to the radiation. Immediately after the exposure, individual rayed animals were isolated, permitted to multiply vegetatively, and a part of the descendants caused to go into autogamy by allowing them to deplete the supply of food rapidly. When it was believed that a culture was in autogamy, a small sample was stained with aceto-orcein and examined. Only those cultures in which at least 90 percent of the sample was in autogamy were ordinarily used. The method is subject to some error in that a small fraction of the animals isolated probably were not in autogamy. For each original rayed animal, 25 autogamous animals were ordinarily obtained. A set of unrayed animals from the same clone was carried through this same procedure as a control.

Each autogamous animal was placed in a limited quantity of culture medium and allowed to multiply for four days. At the end of this period, all were observed for survival and amount of growth. Control animals by this time ordinarily had produced a maximum population which was distinctly starved. The exautogamous descendants of rayed animals frequently showed less growth. The amounts of growth ranged in continuous series from death within the four days through various amounts of growth less than the control to maximum, starved populations just like the controls. For purposes of recording, several qualitative classes were set up, and each exautogamous clone was recorded as belonging to one of these. In the final compilations, all clones in classes which differed distinctly from a maximum, starved population were grouped together as clones of reduced vigor. This included those which failed to survive the four day period; and, at first thought, it might appear that these should be considered as a distinctly separate class. However, many clones which lived for four days proved incapable of surviving further culture. Thus death in four days is not an adequate criterion of ability to live indefinitely and can only be looked upon as one extreme in a continuous series of reduction in vigor.

It is obvious that this method would not be sufficiently accurate to detect very small changes in vigor. A more refined technique using the daily isolation method with daily counting of the number of animals would be expected to detect more changes; and indeed, as will be shown below, this is so. However, the latter method is so laborious that little work has been done with it. The effect of the relative crudeness of the method of detection upon the results will be discussed later in the paper.

As a source of beta radiation, plaques of P^{32} produced in the Oak Ridge pile were used in accordance with the technique described by ANDERSON *et al.* (1947). The animals were placed in 0.5 cc of culture fluid in a thin aluminum dish, a thin aluminum cover was placed over the dish, and it was suspended between two phosphorus plaques in a specially designed lucite plaque holder.

The holder contained three pairs of plaques so that three exposures could be made simultaneously. Dosage calculations were made from measurements of the radiation from the plaques prior to their insertion into the holder. The measurements were made with a plane source ionization chamber calibrated against an extrapolation chamber. The dose per unit time was calculated in r. e. p. from these measurements taking into account the decay of the P^{32}, the absorption by the dish and fluid, and the geometry of the system. There are still some features of this calculation that are not too certain, and so it is possible that the r. e. p. as used in this paper is not the true roentgen equivalent physical. However, some recent work with X-rays on Paramecium has suggested that it is not too badly in error in so far as its biological equivalence to the roentgen is concerned. The two plaques taken together delivered to the animals approximately 13,000 r. e. p. per hour though this figure varied somewhat from time to time. A small amount of gamma radiation was always present as a contaminant.

GENERAL REMARKS UPON THE EFFECTS OF RADIATION

Total doses up to about 13,000 r. e. p. were employed. No noticeable effects of even this highest dose were found as long as the animals multiplied vegetatively. It is possible that there was a slight reduction in fission rate in the first day, but the effect, if any, was so slight that it has not yet been clearly established.

However, when the progeny of rayed animals were sent through autogamy some of the exautogamous clones proved to be of reduced vigor as defined above. This is at least suggestive evidence that mutational changes were induced in the micronucleus by the radiation. Evidence from inheritance studies which supports this belief will be presented in another paper. For present purposes, it will be assumed that reduced vigor after autogamy is evidence for mutational changes in the micronucleus.

THE THEORY OF THE MEASUREMENT OF MUTATION IN PARAMECIUM

The reduction in vigor after autogamy could be and probably is the result of chromosomal aberrations as well as gene mutation, but detailed genetic analysis of any large amount of material would be difficult if not impossible. The one quantity which can be readily measured on a reasonably large scale is the fraction of exautogamous clones which are of reduced vigor. It becomes necessary, therefore, to find out how this quantity is related to the number of mutational changes on various hypotheses concerning the kind and relative frequency of the changes.

The case of gene mutations with individually detectable effects is the simplest and forms the basis for various modifications. I am indebted to Dr. A. S. Householder of Oak Ridge National Laboratory for carrying out the mathematical analysis of this case.

In *Paramecium aurelia*, there are two diploid micronuclei in either or both of which mutations might occur. At autogamy, both micronuclei undergo two meiotic divisions. One of the eight haploid nuclei produced divides mitotically

to form two pronuclei which then fuse to form the diploid synkaryon. From this synkaryon are produced the micronuclei and new macronucleus of the exautogamous clone. The two pronuclei should be of identical genotype since they are formed by the division of a single haploid nucleus, and so the synkaryon which they produce should be completely homozygous.

If a micronucleus is heterozygous for a single mutant gene, the chances that the pronuclei derived from it do not contain the mutant gene should be $\frac{1}{2}$. If the micronucleus contained a independently assorting mutants, the chances that the pronuclei would contain none of them is $(\frac{1}{2})^a$. There is no reason to believe that one micronucleus is any more likely to give rise to the pronuclei than the other. Thus if there were a mutants in one and b in the other, the chances that a synkaryon derived from this animal would have no mutants would be $p(a, b) = (\frac{1}{2})^{a+1} + (\frac{1}{2})^{b+1}$, and the chances that it would contain at least one mutant would be $q(a, b) = 1 - (\frac{1}{2})^{a+1} - (\frac{1}{2})^{b+1}$. As the simplest case, let us assume that the presence of a single mutant gene is sufficient to produce a detectable effect upon vigor. Then the probability of ν normal clones out of n exautogamous clones from an animal with a mutants in one micronucleus and b in the other would be

$$P(\nu/n; a, b) = \binom{n}{\nu} p^{\nu}(a, b) q^{n-\nu}(a, b).$$

The chances that a mutations will occur in a given micronucleus when the mean number per micronucleus is m should be given by the appropriate term of the Poisson series: $p(a) = e^{-m} m^a / a!$. Similarly, the chances of b mutations is given by $p(b) = e^{-m} m^b / b!$.

The chances for ν normals out of n exautogamous clones for a given dose of radiation characterized by a certain value of m is then:

$$P(\nu/n) = \sum_a \sum_b P(\nu/n; a, b) p(a) p(b). \tag{1}$$

The mean number of normal exautogamous animals is:

$$\bar{\nu} = \sum_{\nu}^{1 \cdot n} \sum_a \sum_b \nu P(\nu/n; a, b) p(a) p(b).$$

Substituting the values of the functions on the left and substituting for

$$\sum_{\nu}^{1 \cdot n} \nu P(\nu/n; a, b)$$

its equivalent $np(a, b)$ we have:

$$\bar{\nu} = n \sum_a p(a) \sum_b p(b) p(a, b).$$

Performing the summations over all values of a and b independently, this simplifies to $\bar{\nu} = n\, e^{-m/2}$. Substituting F, the average fraction of exautogamous clones of reduced vigor, for $1 - \bar{\nu}/n$, this becomes:

$$F = 1 - e^{-m/2}. \tag{2}$$

The problem then becomes one of finding F and calculating m from equation (2). It would be possible, at least in theory, to calculate F by the maximum likelihood method using equation (1) as the basis. However, this involves some very complex summations, and it has seemed better to approximate it in another way until it can be shown that the data warrant a more exact treatment. Since it appeared that the maximum likelihood method would yield a treatment dependent upon the values of n and ν, this was done by taking an average over the whole group of rayed animals of the fractions obtained from each using the angular transformation for averaging. Each fraction in the group to be averaged was converted to an angle using the table in SNEDECOR (1946), the arithmetic mean of the angles found, and the resulting mean angle converted back to a fraction. In the rest of the paper, F is calculated in this way.

The expectations for chromosomal aberrations have not been worked out in detail. The expectation of $\frac{1}{2}$ for segregation of gene mutations as embodied in the formula for p(a, b) might have to be changed. If this is done, equation (2) becomes $F=1-e^{-rm}$ where r is some fraction representing the average probability that a synkaryon derived from a micronucleus having an aberration will determine reduced vigor and m is the mean number of chromosomal aberrations per micronucleus.

For gene mutations, further refinements in equation (2) might be desirable to take into account linkage and crossing over. However, except at very high doses, this is probably not important. The most important modification concerns the probability of a single mutant gene producing a detectable effect on vigor. If it be assumed that there are mutants which by themselves have too little effect to be detected but in combination with other similar mutants produce a detectable effect, the following modification has to be made in the development. Let c_0, c_1, c_2, c_3, *etc.* represent the probabilities that animals having respectively none, one, two, three, *etc.* homozygous mutants will be of normal vigor. Let p(a, b, c) represent the probability that an exautogamous clone from an animal having a mutations in one micronucleus and b in the other will be normal. Then this function is equal to

$$c_0\left[\left(\tfrac{1}{2}\right)^{a+1}+\left(\tfrac{1}{2}\right)^{b+1}\right]+c_1\left[a\left(\tfrac{1}{2}\right)^{a+1}+b\left(\tfrac{1}{2}\right)^{b+1}\right]+c_2\left[a(a-1)/2!\left(\tfrac{1}{2}\right)^{a+1}+b(b-1)/2!\left(\tfrac{1}{2}\right)^{b+1}\right]+c_3\left[a(a-1)(a-2)/3!\left(\tfrac{1}{2}\right)^{a+1}+b(b-1)(b-2)/3!\left(\tfrac{1}{2}\right)^{b+1}\right]\quad etc.$$

Substituting this expression in place of p(a, b) in the above development and q(a, b, c) = 1 − p(a, b, c) for q(a, b) and carrying out the analysis as before, we find:

$$F = 1 - e^{-m/2}\left[c_0 + c_1 m/2 + (c_2/2!)(m/2)^2 + (c_3/3!)(m/2)^3 \text{ etc.}\right]. \quad (3)$$

While the solution of this equation is more difficult than that of equation (2), it will be shown below how it can be used.

EFFECT OF STAGE IN THE FISSION CYCLE

Before any adequate data on dosage relations could be obtained, it had to be determined whether radiation was of equal effectiveness at different stages in the life history. So far, no study of this matter has been made for different

parts of the interautogamous interval. Most studies have been carried out by radiating on the eleventh day after a preceding autogamy so that any variation from this source has been for the most part eliminated. Studies have been made on the effectiveness of radiation given at different stages in the fission cycle, and it has been found that radiation is more effective at certain stages than at others.

The method was as follows. A number of animals of a clone were isolated and allowed to multiply with excess food for a day to furnish a good supply of dividing animals as needed. During a ten to fifteen minute period, all animals which were dividing were picked out with a micropipette and put together in a small container. This was repeated at one or more later periods so that two or more groups of animals of known time since the preceding fission were available. At an appropriate time, all groups were exposed simultaneously to the same dose of beta radiation.

The results are shown in table 1. In the last column, the average fraction of exautogamous clones of reduced vigor is shown. The number of rayed or control animals from which these came is given in the sixth column. It is obvious

TABLE 1

Effectiveness of radiation at different stages of the fission cycle.

EXPERIMENT NUMBER	ROW	APPROX. DOSE IN R.E.P.	TIME FROM DIVISION TO BEGINNING OF RADIATION	NOTES	NUMBER OF RAYED OR CONTROL ANIMALS	AVERAGE FRACTION OF EXAUTOGAMOUS CLONES OF REDUCED VIGOR
47.17	1	none	—	—	20	.02
	2	13,000	4 hours	(1)	20	.41
	3	13,000	2 hours	—	20	.88
	4	13,000	10 min.	—	20	.93
47.22	5	none	—	—	10	.005
	6	13,000	4 hours	(2)	12	.41
	7	13,000	4 hours	(3)	18	.96
	8	13,000	1 hour	—	35	.97
47.26	9	none	—	—	11	.02
	10	13,000	4 hours	(4)	13	.21
	11	13,000	1 hour	—	22	.93
47.35	12	none	—	—	68	.06
	13	6,500	5½ hours	(2)	16	.29
	14	6,500	5½ hours	(3)	14	.92
	15	6,500	4½ hours	(4)	30	.10
	16	6,500	2 hours	—	15	.81

Notes: (1) Both divided and undivided animals present at end of radiation. Not separated.
(2) Both divided and undivided animals present at end of radiation. This group includes only the undivided ones.
(3) Both divided and undivided animals present at the end of radiation. This group includes only the divided ones.
(4) No animals had divided by the end of radiation.

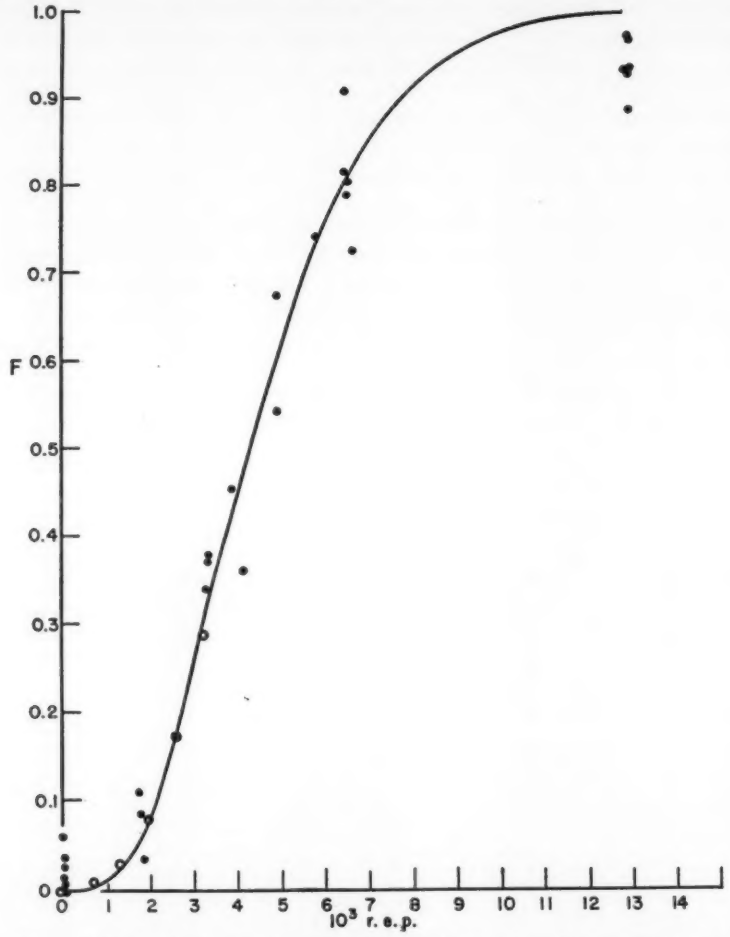

FIGURE 1. The relation between dose of beta radiation in 10^3 r. e. p. and the average fraction of exautogamous clones of reduced vigor (F). The solid points represent single exposure experiments; the open circles, fractionated exposures. Each point represents the average for about 20 rayed animals. The curve was calculated from an equation to be explained in the text.

that radiation at different stages in the fission cycle was of markedly different effectiveness in producing detectable changes. Animals rayed just prior to the next division (rows 2, 6, 10, 13, 15) produced far fewer clones of reduced viability than did those rayed immediately after fission (4) or one or two hours afterwards (3, 8, 11, 16). A comparison of rows 6 and 7 and of rows 13 and 14 shows that the ineffective period must end rather abruptly just at or just before the beginning of fission. In each case, the two rows represent single groups of animals which divided some four to five hours before radiation. Just

before radiation, few if any animals in these groups had divided, but during the half hour to hour needed for radiation a part of them did as indicated by their small size and also by the dividing animals seen immediately after radiation. On isolation, a record was kept as to whether the animal had divided or not as judged by its size. On the basis of this record, the divided and undivided groups were considered separately in analyzing the results. Those which had divided showed markedly more effect than those which had not.

The results indicate that radiation given in a period of an hour or more before fission is relatively ineffective when compared to the same exposure given during fission and for at least two hours afterwards. There is a suggestion of

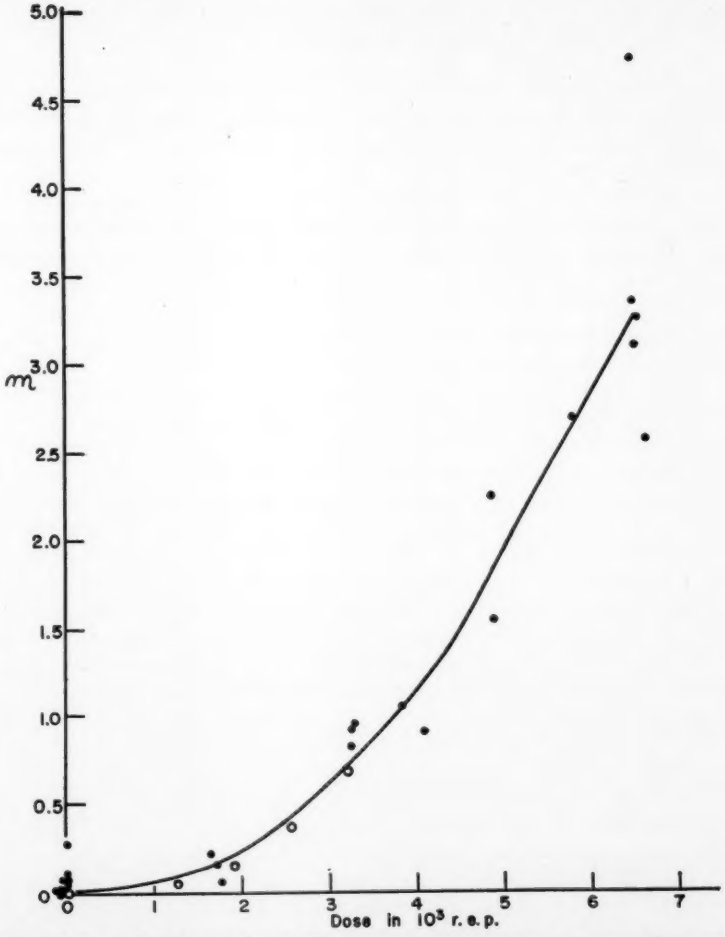

FIGURE 2. The relation between dose in 10^3 r. e. p. and m calculated from the relation $F=1-e^{-m/3}$. The individual points are the same as plotted in figure 1.

a slight dropping off in effectiveness as the time since fission increases but this cannot be considered as established. All work to be reported below was done with animals one or two hours after fission.

DOSAGE DEPENDENCE

Figure 1 shows the relation between the average fraction of exautogamous clones of reduced vigor, calculated as indicated above, and dose; and figure 2 shows the relation between m as calculated from equation (2) and dose. The points for the individual experiments are based, in most cases, upon the progeny of 20 animals with approximately 25 exautogamous clones from each. The open circle points representing fractionated doses will be discussed later. Only doses up to 6500 r. e. p. are included in figure 2 since the very high values of F obtained at 13,000 r. e. p. make it impossible to calculate m with any precision.

On the simple gene mutation hypothesis, m should increase as a linear function of dose. It is obvious that this is not the case. A curve has been fitted to the data by eye, and it appears to approximate an increase of m as some power of the dose.

FRACTIONATED DOSES

Since the relation of m to dose is not linear, a reexamination of the simple gene mutation hypothesis is necessary. Two alternatives seem possible. If chromosomal aberrations of the so-called two-hit type make up most of the detectable changes, then m, as the mean number of aberrations, would be expected to increase approximately as the square of the dose. On the other hand, if most of the reduction in vigor is the result of the combined action of mutant genes with individually small effects, then m as calculated from equation (2) would be expected to show a non-linear relation to dose even though the true mean number of gene mutations as calculated from an appropriate form of equation (3) should show a linear relation.

It should be possible to distinguish between these two hypotheses by use of fractionated doses. Various workers (see LEA 1947, for review) have shown for Tradescantia that a given dose of radiation is less effective in producing interchange aberrations if it is given at low intensity over a long time than at high intensity over a short time. SAX (1941) has shown that a dose is less effective when given in fractions with a rest period in between than when given as a single concentrated exposure. These facts have been interpreted to mean that broken ends remain available for recombination for only relatively short periods of time.

For Paramecium, it seems highly improbable that breaks produced on one day would remain available for recombination with breaks produced on the following day after some four to five fissions. Thus if the non-linear relation of m were due to two-hit aberrations, a dose given in small daily fractions would be expected to be much less effective than the same total dose given within a single interfission interval; and a linear relation to dose should be approached. On the other hand if the non-linear relation is the result of most of the detectable reduction in vigor being due to the combined effect of several genes, fractionation of the dose should have no effect.

The experiment was performed as follows. A number of animals from a single clone were chosen and divided into two groups. One group was kept as a control and the other was exposed to about 300 r. e. p. After radiation, the animals were isolated and allowed to multiply. One descendant from each was chosen on the next day, exposed to 300 r. e. p., and reisolated. On the next day, one descendant of each was isolated and its progeny kept without further radiation for the remainder of the experiment. This group, then, received a total of 600 r. e. p. in two fractions. At the same time another descendant of each of the animals which had received 600 r. e. p. was chosen, exposed again to 300 r. e. p., and reisolated. This procedure was repeated every day for ten days, every second day a group of animals being set aside and kept without further radiation. Thus groups were obtained which had received approximately 600, 1200, 1800, 2400, and 3000 r. e. p. in 300 r. e. p. daily doses. At the end of the ten day period, autogamous animals were obtained from 20 lines of each group, including the control.

The results are plotted in figures 1 and 2 as open circles. It is obvious that fractionation of the dose made little if any difference in the results. It can be concluded that two-hit aberrations could not have contributed much to the effect. This leaves as the most plausible hypothesis that most of the detectable reduction in vigor is due to the combined action of several mutant genes with individual effects too small to be detected by the usual method.

DEMONSTRATION OF SMALL REDUCTIONS IN VIGOR

If this is so, a number of clones classified as normal by the usual test should be homozygous for mutant genes with very slight effects upon vigor which might be detected by a more refined method. To find out if this were so, ten animals from a clone were exposed to 6500 r of 250 KV X-rays. Ten animals from the same clone were kept without radiation as controls. Of the 250 exautogamous clones from rayed animals, 50 were classified as normal after the standard four day test. These were distributed among the progeny of all ten rayed lines. A single animal was isolated from 48 of these apparently normal clones, and at the same time an animal was isolated from 17 normal exautogamous clones from the controls. The descendants of these 65 animals were kept as daily isolation lines with daily counts of the number of animals for five days. Of the 48 clones from rayed animals, 39 had a distinctly lower fission rate than any of the controls. In addition, a number of abnormal animals appeared and 14 of the clones died out before the end of the period. Thus a more refined technique demonstrates that a large number of changes in vigor occur which are not detected by the standard method. The fact that some of the clones which had appeared normal at four days died within the subsequent five day period suggests that the failure to detect all changes by the usual method may be due in part to lag phenomena. Some of the mutant genotypes may have a lag in their expression lasting beyond the four day period. However, this is not the only cause of the failure to detect all changes; for several of the clones continued through the five day period with a constant fission rate only about one fission per day less than the controls. It is highly improbable that such clones would be detected by a four day total growth test no matter

how long after autogamy it was made. In addition, it seems not unlikely that, other things being equal, the more deleterious mutants present, the shorter the lag period.

MUTATION RATE

Equation (3) can be solved for m if the constants c_0, c_1, c_2, *etc.* can be found and if the relation $m = bD$; that is, a linear relation to dose, is assumed. This last assumption seems reasonably justified since m is by definition the mean number of gene mutations per micronucleus, and from the work with other organisms the number of mutations should be a linear function of dose.

It is necessary, then, to estimate the value of c_0, c_1, c_2, *etc.* and of b from the data. This could be done by fitting equation (3) to the data by the least squares method, but the complex form of this equation makes this approach very tedious. It is probable that the data are not accurate enough to justify the labor involved. For this reason, less accurate approximation methods were used. Two methods were tried which gave slightly different values for the constants. Quite good fits can be gotten when c_0, c_1, c_2, c_3 are equal to unity; c_4 is either unity or zero; and c_5, c_6, *etc.* are zero. The value of b is approximately .002. These values mean that some four to five mutants must be present in an exautogamous clone to produce a detectable effect on vigor and that approximately two mutations affecting this character are produced per micronucleus per 1000 r. e. p.

The curve in figure 1 was drawn with c_0, c_1, c_2 and c_3 equal to unity and b equal to .0017. Most of the points fall fairly close to the line except those at about 13,000 r. e. p. In the section on methods, it was pointed out that a small error was inherent in the technique in that occasional non-autogamous animals were isolated and treated as though they were autogamous. Such animals would probably produce a four-day culture which would be classified as normal. This error would have little effect until the fraction of clones of reduced vigor becomes very high. At the upper extreme, it would have the effect of making the experimental value of F approach some value less than unity as an upper asymptote.

DISCUSSION

A high resistance of vegetatively multiplying paramecia to ionizing radiation has been reported, among others, by BACK and HALBERSTAEDTER (1945) and is, perhaps, not too surprising. The presence of the macronucleus, according to SONNEBORN (1940) a multiple structure with each gene represented many times, makes it improbable that gene mutations induced in vegetatively reproducing animals would express themselves unless doses capable of causing tremendous numbers of mutations were applied. Thus the animal is well protected against the effects of gene mutations and immediate deleterious effects of radiation would have to be produced through some other mechanism which might well be less readily affected by radiation than are the genes. The fact that, even with relatively very small doses, mutational effects appear after autogamy emphasizes the need to control this process in studies of the effect of radiation on Paramecium.

The difference of effectiveness of radiation applied at different stages of the fission cycle remains at present unexplained. It should be noted that the ineffective period probably includes, if it does not coincide with, the division of the micronuclei which immediately precedes cytoplasmic division; but it is not clear what interpretation to place upon this apparent connection.

It is not possible by the methods used in this work to detect individual mutations. However, by making a few simplifying assumptions, it is possible to arrive at an approximate value for the overall mutation rate including as a major component mutants with individually undetectable effects. The approximate values given in the preceding section for the constants of equation (3) allow for only one class of mutants, those which express themselves when four or more are together and do not express themselves when three or less are present. It is obvious that this is an oversimplification. There are undoubtedly mutants which express themselves in the absence of any others, and the expression of those that do not, probably depends not only upon the number, but also the kind of other mutants present. Moreover, it seems probable that chromosomal aberrations by their segregation from the heterozygous state add to the total reduced vigor found. The data merely show that two-hit chromosomal aberrations and gene mutations with large effects do not make a very considerable contribution to the changes detected. While the major part of the effect has been attributed to gene mutations, it is obvious that nothing in the data rules out the possibility that very short deletions with small effects on vigor may be involved. It seems improbable that deletions that were not very short could play an important role since they would be expected to have major effects on vigor.

The calculated value of about two mutations per micronucleus per 1000 r. e. p. is based on the assumption that there is only one class of mutants, those which have an effect only when four or more are present. Mutants which express themselves when one, two, or three are present will tend to make the calculated rate too high while those which express themselves only when five, six, or more are present will tend to make it too low. Insofar as these opposing errors cancel each other out, the calculation will be correct.

The present investigation confirms studies on other organisms that show that mutations with very slight effect are the commonest kind. MULLER (1941) states that about twice as many "detrimental" mutations are produced as lethals in Drosophila. In Paramecium, it would appear that the ratio is considerably greater perhaps because the method used detects, at least in part, mutations with very slight effects.

SUMMARY

1. Beta radiation from an external P^{32} source in doses up to 13,000 r. e. p. has no immediate marked effect upon vegetatively multiplying *Paramecium aurelia*.

2. When the progeny of rayed animals are sent through autogamy, many of the exautogamous clones are of reduced vigor as judged by total growth in four days.

3. This reduced vigor is interpreted as the expression of mutational changes

in the micronuclei partly because it first appears when the animals are made homozygous by autogamy, partly because data on inheritance to be published in a separate paper support this view.

4. Radiation given in a period of about an hour before cytoplasmic division is less effective than radiation given at other stages of the fission cycle. No adequate explanation is available for this phenomenon.

5. The relation of the percentage of exautogamous clones of reduced vigor to dose shows that most of the mutational changes cannot be gene mutations with individually detectable effects. The relation is consistent either with the hypothesis that most of the effect is due to two-hit chromosomal aberrations or with the hypothesis that most of it is due to the combined action of several mutant genes with individually undetectable effects.

6. Fractionation of the dose has no noticeable effect on dosage relations so two-hit chromosomal aberrations cannot be an important factor.

7. This leaves the hypothesis that most of the detectable reduction in vigor is due to the combined effect of mutant genes with individual effects too small to be detected.

8. This hypothesis gives expectations in agreement with the dosage relations and is further supported by the fact that a number of clones which appear normal by the usual test for vigor prove to be of reduced vigor when examined by a more refined method.

9. The mathematical relation between the average fraction of exautogamous clones of reduced vigor and the mean number of mutations per micronucleus has been worked out for this hypothesis. By approximation calculations, a value of about two mutations per micronucleus per 1000 r. e. p. has been obtained.

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